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The Myosin-Binding UCS Domain but not the Hsp90-Binding TPR Domain of the UNC-45 Chaperone is Essential for Myosin Accumulation and Assembly in *Caenorhabditis elegans*

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by

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Dedication

To my family

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The Myosin-Binding UCS Domain but not the Hsp90-Binding TPR Domain of the UNC-45 Chaperone is Essential for Myosin Accumulation and Assembly in *Caenorhabditis elegans*

Publication No._____

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The University of Texas Medical Branch, 2011

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The UNC-45 family of molecular chaperones is expressed in metazoan organisms from *C. elegans* to humans. The UNC-45 protein is essential in *C. elegans* for early body-wall muscle cell development and A band assembly. We show that the myosin-binding UCS domain of UNC-45 alone is sufficient to rescue embryonic lethal *unc-45* null mutants arrested in embryonic muscle development and temperature-sensitive loss-of-function *unc-45* mutants defective in worm A band assembly. Removal of the Hsp90-binding TPR domain of UNC-45 does not affect rescue. Similar results were obtained with overexpression of the same fragments in wild-type nematodes when assayed by diminution of myosin accumulation and assembly. Titration experiments show that on a per molecule basis, UCS has greater activity in *C. elegans* muscle *in vivo* than full-length UNC-45 protein, suggesting it may be inhibited by either the TPR domain or its interaction with the general chaperone Hsp90. *In vitro* experiments with purified

recombinant *C. elegans* Hsp90 and UNC-45 proteins show that they compete for binding to *C. elegans* myosin. Our *in vivo* genetic and *in vitro* biochemical experiments are consistent with a novel inhibitory role for Hsp90 with respect to UNC-45 action.

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Chapter 1: Background and Introduction

MYOSINS

Myosin is a superfamily of protein motors that play essential roles in actin-based cell motility. More than 24 classes of myosins have been discovered in multiple eukaryotic species on the basis of their conserved motor domains that exhibit their own specialized structures and related functions (Foth *et al.* 2006). Class II, or conventional myosins, function in a broad spectrum of essential cellular processes, including cytokinesis during cell division and muscle contraction (Sellers 2000). Class II myosins are the largest and most extensively investigated group. Other classes of myosins are termed unconventional myosins. Among unconventional myosins, classes I and V are the most studied groups. Unconventional myosins have important functions in various cellular processes, including cellular trafficking, phagocytosis, and polarized cell growth (Berg *et al.* 2001; Oliver *et al.* 1999).

Generally, all myosins have three regions: a conserved N-terminal motor domain (myosin head) for the binding and hydrolysis of ATP and actin binding, a neck domain where myosin light chains bind, and a C-terminal class specific tail domain to position motor domains. The neck domain also binds calmodulin through its one or more IQ motifs. Different classes of myosin contain distinct tail domains, varying greatly in sequence and length. Differential tail domains are considered to have an important function to distinguish class specific roles (Oliver *et al.* 1999).

Class II Myosin

Class II (conventional) differs from the other myosin classes because it is the only class that assembles into bipolar thick filaments through the self-association of the C-terminal tail region. Class II myosins are hexameric proteins consisting of two heavy chains, two essential light chains, and two regulatory light chains (**Fig. 1.1**). Every myosin II molecule assumes a two headed structure because of the dimerization of the

heavy chains through the C-terminal coiled coil rods. Myosin II can be cleaved into its distinct parts by proteases. Chymotrypsin cleaves myosin II at the base of the neck, leading to the generation of heavy meromyosin (HMM) bearing the head and neck regions and light meromyosin (LMM). LMM is a rod-like tail region, consisting of an α–helical coiled coil. HMM can be farther digested into subfragment 2 or S2 (the neck region) and subfragment 1 or S1 (two single heads) by papain (**Fig. 1.1**).

C. elegans Class II Myosin

The assembly and organization of myosin II into *C. elegans* muscle thick filaments is the focus of this study. *C. elegans* has served as a model for studying the structure and assembly of thick filaments because of well established genetic, biochemical and structural backgrounds.

There are four differential muscle myosin heavy chain (MHC) genes which encode four distinct class II myosin heavy chains in *C. elegans* (Miller *et al.* 1986). Myosin heavy chain A and myosin heavy chain B mainly exist in body-wall muscle cells, whereas myosin heavy chain C and myosin heavy chain D are solely expressed in the pharynx (Mackenzie *et al.* 1978). The genes *myo-3*, *unc-54*, *myo-2*, and *myo-1* encode these four myosin II heavy chain isoforms respectively (Brenner 1974). Myosin A and myosin B are co-expressed in every *C. elegans* body-wall muscle cell (Ardizzi and Epstein 1987; Miller *et al.* 1983). Myosin C and myosin D are also called pharyngeal myosins due to the exclusive existence in the pharynx.

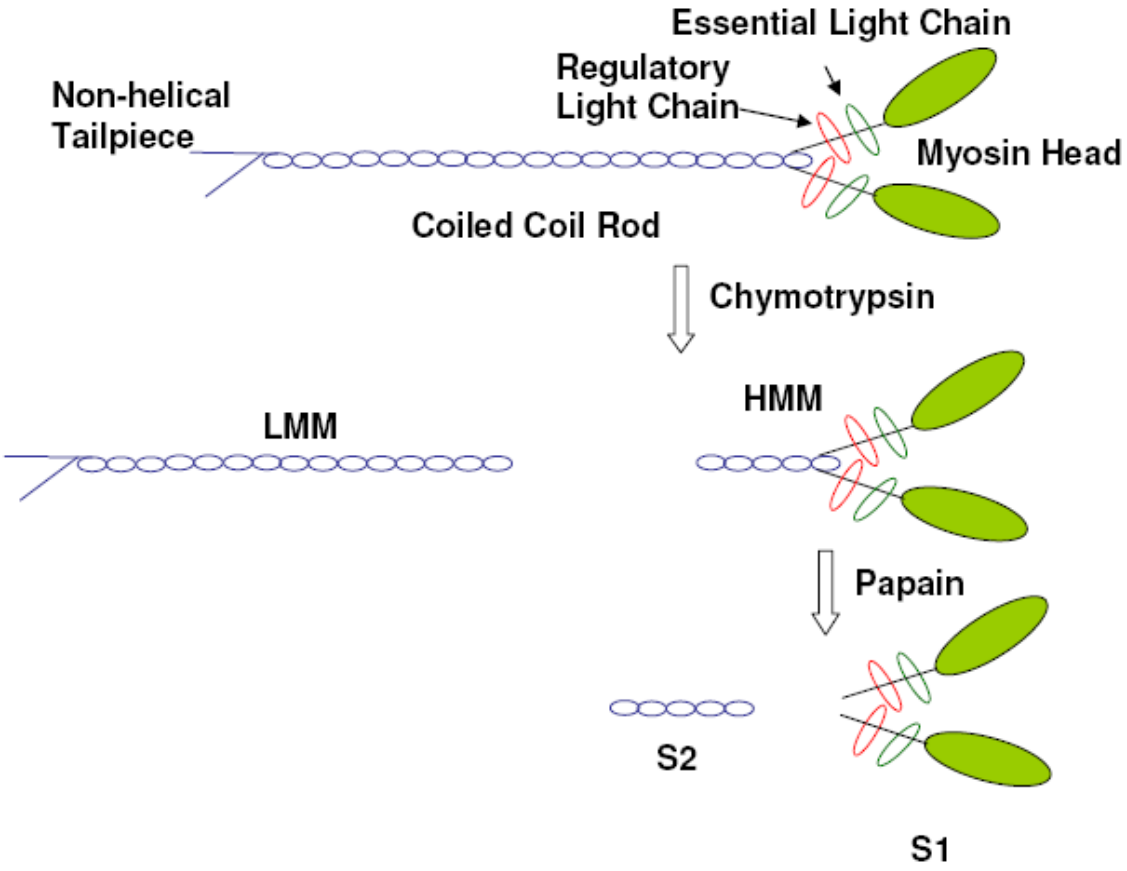


Figure 1.1: Myosin II and its proteolytic digestion. Myosin II is a hexameric complex, which is composed of two heavy chains and two pairs of essential and regulatory light chains. The heavy chains dimerize through the coiled coil rod and then assemble into filamentous structures. Myosin II molecule can be digested into heavy and light meromyosin (HMM and LMM, respectively). HMM can be farther digested into subfragments 1 and 2 (S1 and S2, repectively). LMM is the filament-forming portion of the myosin rod domain.

ASSEMBLY OF CLASS II MYOSIN INTO SARCOMERE

Class II myosin is the main element of thick filaments in the muscle sarcomere and sarcomere is the basic unit in muscle cells. The coiled coil rod (the carboxyl-terminal part of the myosin heavy chain to the head) permits dimerization of heavy chains to form myosin and subsequent assembly of myosins into thick filaments.

The rod region has been recognized as the filament-forming domain of the myosin heavy chain for a long time (Squire 1981). The α-helical coiled coil is based on a repeating seven residue unit within each myosin heavy chain molecule, a-b-c-d-e-f-g. Among these seven residues, residues a and d usually have hydrophobic side chains (McLachlan and Stewart 1975; McLachlan and Karn 1983). A longer range 28-reisude repeat of interposed acidic and basic amino acid side chains was also found to form salt bridges between neighboring α-helical coiled coils (Dibb *et al.* 1989; McLachlan and Karn 1983). The assembly competent domain, or ACD, was defined by a C-terminal 29-amino-acid sequence in a conserved 63-residue region of the myosin rod (Cohen and Parry 1998; Sohn *et al.* 1997). ACD is critical for assembly of myosin or rod subfragments into structures with higher-order (Sohn *et al.* 1997). ACD was presumed to function as a "trigger sequence" to align the paired helices of the myosin heavy chains in parallel to boost the organization of the coiled coil (Krammerer *et al.* 1998).

Vertebrate Striated Muscle Sarcomere

A sarcomere is defined as the segment between two neighboring Z-lines, which appear as a series of dark lines in electron micrographs of vertebrate cross-striated muscle (**Fig. 1.2A**). The vertebrate striated muscle sarcomere is composed of A bands and I bands. The region of I band surrounds the Z-line. A band follows I band. A band contains both thick filaments (myosin filaments) and thin filaments (actin filaments) at the same time, while there are only thin filaments in I bands. The paler region within A band is

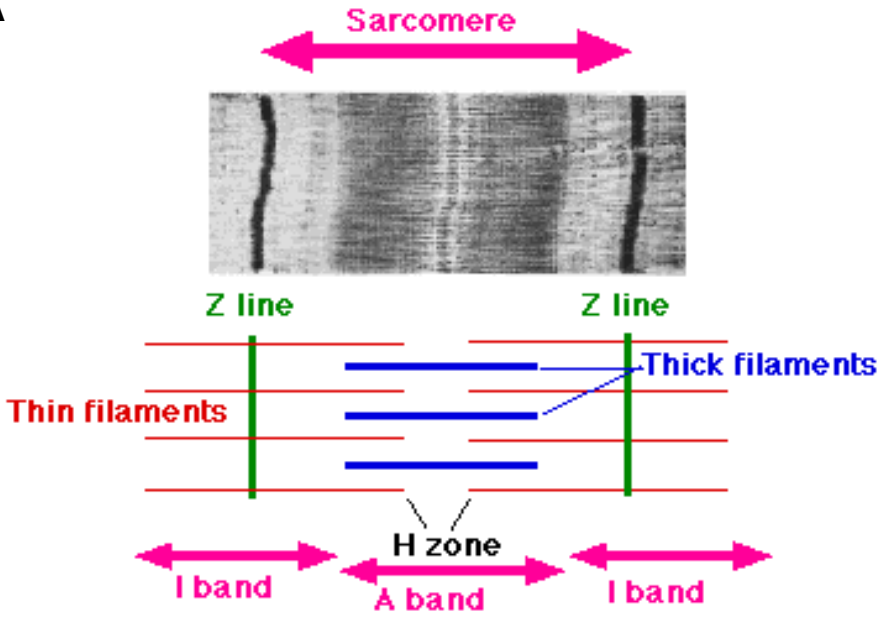
called H zone (**Fig. 1.2A**). The interaction between actin and myosin filaments in A band of the sarcomere is responsible for the muscle contraction.

C. elegans Body-Wall Muscle Sarcomere

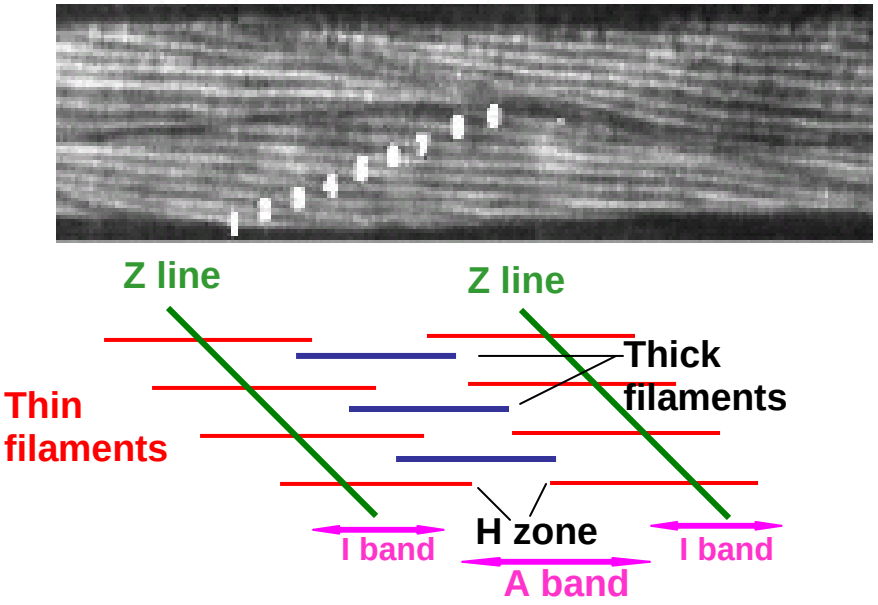
The *C. elegans* body-wall muscle sarcomere consists of A bands and I bands like vertebrate striated muscle sarcomere (**Fig. 1.2B**). A band is defined as the area of interdigitation between thick filaments and thin filaments. I band is composed of the thin filaments alone. Thick filaments are fixed at the M-band in the middle of H zone, whereas thin filaments are cross-linked by the Z-line. Polarized light microscopy is a powerful way to study *C. elegans* body-wall muscle. Thick filaments are light and thin filaments are dark (**Fig. 1.2B**). There is an about 8 degree oblique in *C. elegans* body-wall muscle sarcomeres, compared to vertebrate striated muscle sarcomeres.

Figure 1.2: Sarcomere assembly of vertebrate striated muscle and *C. elegans* body-wall muscle. (A) Electron micrograph and cartoon explanation of vertebrate striated muscle sarcomere. A band is characterized as the area of interdigitation between thick filaments and thin filaments. I band merely consist of the thin filaments. Thick filaments are fixed at the M-band in the middle of H zone, while thin filaments are cross-linked by the Z-line. (B) Polarized light microscopy and cartoon explanation of *C. elegans* body-wall muscle sarcomere. Thick filaments are light, whereas thin filaments are dark under polarized light microscope. *C. elegans* body-wall muscle sarocmeres have an approximate 8 degree oblique compared to vertebrate striated muscle sarcomeres.

A


Sarcomere
Z line
Z line
Thick filaments
Thin filaments
H zone
I band
A band
I band

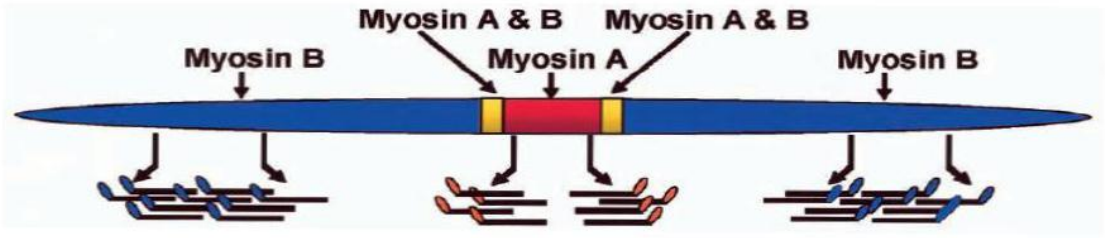
B


Z line
Z line
Thick
filaments
Thin
filaments
I band
H zone
I band
A band

Structure of the C. elegans Thick Filament and Functions of Myosin A and Myosin B in the C. elegans Thick Filament

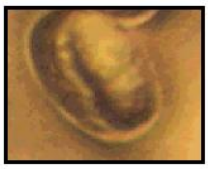
The detailed structure of the *C. elegans* body-wall muscle thick filament is necessary for unveiling the myosin assembly process. The *C. elegans* body-wall muscle thick filament is a bipolar structure consisting of proteins assembled into different regions. Myosin heavy chain A and myosin heavy chain B are mainly expressed in body-wall muscle cells together. Although myosin A and myosin B are very similar in primary sequence, there are significant functional differences between them. Myosin A and myosin B are 65% identical and 79% similar in amino acid sequence. The major differences of functional significance of myosin A and myosin B lie in the rod domain. The rod domain of myosin A contains two regions, a region containing 264 amino acids in the center and a region containing 170 amino acids at the C-terminus, which are sufficient for providing the crucial function of myosin A (Hoppe and Waterston 1996).

The arrangement of myosin A and myosin B provides more insight into the functional differences of each myosin. Myosin A and myosin B have distinct distribution patterns along the thick filament (Miller *et al.* 1983; Schachat *et al.* 1977; Schachat *et al.* 1978). Myosin A is found only within the 1.8 μ m region in the center of the thick filament while myosin B is found in the polar arms of the thick filament (**Fig. 1.3**). Myosin A represents roughly 20% of the total body-wall muscle myosins, whereas myosin B composes about 80% of the total body-wall muscle myosins. The middle of the bipolar central region is the “bare zone”, where there are no myosin heads ornamenting the thick filament. There are two little flanking zones on both sides of the central region, where myosin A and myosin B co-assemble (Miller *et al.* 1983).

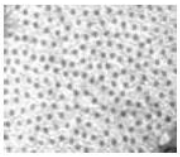


(Landsverk and Epstein, CMLS 2005)

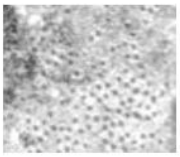
Myosin A null mutant:
Paralyzed, arrested at twofold (Pat)



Myosin B null mutant:
Uncoordinated (Unc)



Wild-type



Myosin B
null mutant

Figure 1.3: Organization and functions of myosin heavy chains in *C. elegans* thick filaments. Myosin A localizes within the 1.8 μm region in the center of the thick filament and myosin B flanks on both sides in the polar arms of the thick filament. Myosin A and myosin B simultaneously exist in an area on both sides of the myosin A-containing region in the center. Myosin A is packed in an anti-parallel manner to generate a bare zone in the center of the thick filament. Myosin B assumes a parallel manner to generate the polar arms. Worms missing myosin A are arrested at the embryonic two-fold stage and they can not elongate, which is called *Pat* (paralyzed, arrest at twofold) phenotype (Williams and Waterston 1994). In contrast, worms lacking myosin B can survive but they are severely paralyzed with a decreased number of myosin filaments, composed completely of myosin A, which is called *Unc* (uncoordinated) phenotype.

The central region is considered to contain the initiation site for the occurrence of thick filament assembly. Myosin A pack in an anti-parallel tail-to-tail manner in the central region and myosin B organizes itself in a parallel manner to neighboring sides of this polar central region (**Fig. 1.3**). The capability of myosin A molecules to assemble in an anti-parallel manner in the center of the thick filament must be conferred by its tail region and this function is thought to be essential for the beginning of thick filament assembly (Miller *et al.* 1983).

The differential localization of myosin A and myosin B suggests that they play different roles in the assembly of thick filaments. Genetic studies have proved the distinct functions of myosin A and myosin B in the assembly and organization of *C. elegans* thick filaments (**Fig. 1.3**). Worms deficient in myosin A arrest and then decease during embryonic development around the two-fold stage, which is called *pat* (paralyzed, arrest at two-fold). Analyses of the arrested embryos by immunofluorescence and electron microscopy have revealed inappropriate muscle development with severely damaged thick filament assembly (Waterston 1989). The phenotype of worms deficient in myosin A indicates a function of myosin A during the beginning assembly of the thick filaments. Unlike myosin A, worms missing myosin B can go through the development but they are severely paralyzed, which is called *unc* (uncoordinated) (Epstein *et al.* 1974). These worms have disrupted myofibril organization and a decreased number of thick filaments consisting solely of the myosin A isoform (Epstein *et al.* 1986; Macleod *et al.* 1977; Mackenzie and Epstein 1980). The phenotype of worms lacking myosin B suggest that myosin A dimers are also able to pack in parallel fashion besides the anti-parallel manner exhibited in the center of the thick filament. The anti-parallel manner of myosin A assembly has been confirmed by the observation that the *unc* phenotype of myosin B null worms can be suppressed by overexpression of myosin A via introduction of a *myo-3*

transgene (Fire and Waterston 1989) or by chromosomal duplication of *myo-3* (Maruyama *et al.* 1989; Otsuka 1986; Riddle and Brenner 1978; Waterston *et al.* 1982). The inverse is not right as overexpressing myosin B could not rescue the *pat* phenotype of myosin A null worms (Waterston 1989). Therefore, it has been indicated that myosin A serves an essential role that can not be substituted by myosin B. Regulation of myosin B expression levels also appears to be significant for proper muscle development because overexpression of myosin B causes problems in muscle structure and disorganizes the distribution of myosin A and myosin B in wild-type worms (Fire and Waterston 1989).

All Myosin Domains are Indispensable for Appropriate Function in C. elegans

Genetic studies have indicated that the whole myosin II molecule is essential for appropriate organization of myosin containing structures. The interaction between the myosin motor domain and its partnering proteins (such as actin) is necessary for myosin assembly and organization. The interaction of the myosin neck region with essential and regulatory light chains is also critical although the complete tail region is essential and enough for assembly of myosins into myosin-involved structure.

Over 40 dominant-negative mutations in the *C. elegans unc-54* locus (*unc-54(d)*) have been identified (Bejsovec and Anderson 1990). These dominant-negative mutations are missense alleles, affecting the globular head of myosin. Recessive negative mutations in *unc-54* (*unc-54(0)*) also have been discovered (Bejsovec and Anderson 1988). These recessive mutations cause the premature polypeptide chain termination in MHC B. The phenotypes of various *unc-54* genotypes are quite different (**Table 1.1**).

Table 1.1: Various *unc-54* genotypes generate differential phenotypes

Genotype	Motility Phenotype
+/+	wild type
unc-54(0)/+	wild type
unc-54(0)/ unc-54(0)	paralyzed
unc-54(d)/+	paralyzed, severity varies, depending on allele
unc-54(d)/ unc-54(d)	late embryonic or early larval lethal
unc-54(d)/eDf10	either severely paralyzed or lethal, depending on allele
unc-54(d);sup-3/ unc-54(d);sup-3	either severely paralyzed or lethal, depending on allele

(modified from Bejsovec and Anderson 1988)

unc-54(0) mutants' phenotypes are typical for alleles that do not express MHC B. *eDf10* is a deletion that eliminates the entire *unc-54* gene and it is recessive to wild type. *sup-3* overexpresses MHC A about twofold, which has a duplication of the *myo 3* gene (Dibb *et al.* 1985; Epstein *et al.* 1974; Maruyama *et al.* 1989; Waterston *et al.* 1982; Zengel and Epstein 1980).

All dominant-negative mutations lie in the myosin head affecting myosin ATP-binding site and actin-binding site (Bejsovec and Anderson 1990). Heterozygous dominant-negative *unc-54* mutant worms are highly paralyzed. Mutant myosin B cannot assemble into the thick filaments and these mutant myosins further impair the assembly of wild-type myosin B and myosin A into the thick filaments in heterozygous dominant-negative *unc-54* mutant worms (Bejsovec and Anderson 1988). The accumulation of wild-type MHC A and MHC B is not affected in heterozygous *unc-54* mutant worms although the assembly of wild-type MHC A and MHC B is disrupted by the mutant MHC B (Bejsovec and Anderson 1990). This phenotype can be generated even by low amounts of the mutant myosin B since mutant protein is not stable *in vivo*. Earlier research has indicated that the existence of mutant myosin B can lead to complete disruption of thick filament assembly during *C. elegans* body-wall muscle development even though the accumulation level of mutant myosin B is no more than 2% of wild-type myosin's amount (Bejsovec and Anderson 1988). This dominant-negative phenotype of mutant myosin B indicates that the myosin motor domain is critial for the appropriate organization and assembly of thick filaments as expression of mutant myosin B exhibits severer consequence than knockout of myosin B.

The interaction of residues in the myosin tail region has an important function in the assembly of structural organization of thick filaments. The required regions of *C. elegans* myosin A for the proper start of the thick filament assembly lie in its rod domain, which cannot be complemented by the equivalent parts of the tail region from myosin B (Hoppe and Waterston 1996). Four residues on the terminus of the myosin A tail region have been shown to be necessary for appropriate assembly of thick filaments during the early nematode development stages (Hoppe *et al.* 2003). Constructs without these four residues and the tailpiece of the rod can rescue the deficiency of myosin A or myosin B.

However, these constructs cannot rescue worms lacking both isoforms at the same time. Delayed onset of movement and myosin localization has been observed for embryos expressing only truncated myosin heavy chains. In addition, these embryos arrest and die at the embryonic twofold stage (Hoppe *et al.* 2003). Therefore, it is quite important to have an entirely functional myosin tail region for thick filament assembly, organization and development in *C. elegans*.

Additional Proteins in C. elegans Myosin Organization

The nematode sarcomere contains not only class II myosins but also other proteins, such as paramyosin and filagenins. Paramyosin and filagenins are critical for the regulation of assembly, organization, and structural stability of thick filaments in *C. elegans* sarcomere.

Paramyosin is another major component of the *C. elegans* thick filament. The gene *unc-15* encodes paramyosin in *C. elegans*, which has about 38% identical in residue sequence, compared to the tail region of class II myosin heavy chain (Harris and Epstein 1977; Kagawa *et al.* 1989; Waterston *et al.* 1977). Paramyosin has 872 amino acids and it is expressed in all worm muscle cells (Ardizzi and Epstein 1987). Paramyosin has the same features as the tail region of a myosin heavy chain with coiled-coil domains throughout most of its length, containing the first and four residues in a seven (heptad) residue repeat and the longer 28-residue repeat (Kagawa *et al.* 1989).

Paramyosin and myosin A have similar amount of hydrophobicity and glycine content, which indicates that paramyosin assembles in an anti-parallel manner as myosin A into the thick filament (Hoppe and Waterston 1996). Paramyosin has been deemed as the scaffold for the assembly of myosin A and myosin B. Worms without paramyosin are severely paralyzed and they contain thick filaments with core-like structures (Epstein *et al.* 1986; Waterston *et al.* 1977). The absence of paramyosin does not affect myosin A

but changes the synthesis, accumulation and assembly of myosin B (Mackenzie and Epstein 1980; White *et al.* 2003). Thick filaments in paramyosin deficient worms are not stable and myosin B colocalizes with myosin A in the center of thick filaments. Therefore, myosin B is able to pack in an anti-parallel manner like myosin A in the presence of paramyosin. Based on above observation, it is hypothesized that myosin A assembles first in the central region to initiate the thick filament assembly, elongation of the paramyosin core comes after, and then myosin B assembles to the poles (Epstein *et al.* 1985).

Isolated thick filaments can be gradually solubilized from the arms inward to the central region by increasing salt concentrations. The first solubilized protein is myosin B, followed by myosin A, and then around 70% of the total paramyosin. An indissoluble part (core) is produced after applying high salt to isolated thick filaments, composed of myosin A and the other proteins (Epstein *et al.* 1985). This core is supposed to serve as the template to assemble thick filament proteins (Epstein *et al.* 1988). The study of the core resulted in the discovery of the filagenins as core-associated proteins (Deitiker and Epstein 1983; Liu *et al.* 1998; Liu *et al.* 2000).

Three filagenin isoforms (α, β, and γ) have been discovered. They are extremely basic (α-filagenin, 30kDa and a pI of 9.67; β-filagenin, 28 kDa and a pI of 9.73; γ-filagenin, 20kDa and a pI of 10.76). Filagenins are only found within the thick filament core structure. They have different expression pattern and differential localization along thick filament. α-filagenin has a myosin A-like central region localization, whereas β- and γ-filagenins show myosin B-like polar localizations (Liu *et al.* 1998; Liu *et al.* 2000). The differential localization of filagenins might perform an essential function in regulation of thick filament assembly and stability. Filagenins probably function as

coupling proteins to tether the core paramyosin subfilaments to make it rigid (Muller *et al.* 2001).

CHAPERONES INVOLVED IN MYOSIN FOLDING AND ASSEMBLY

The folding pathway of the myosin molecule is not clear yet. Previous studies have shown that proper myosin folding and assembly need to have additional proteins to function in the myosin folding and assembly pathway in addition to above mentioned sarcomere components.

Recombinant-expressed myosin tails and myosin light chains in bacteria are in native state and can be soluble in high salt (De Lozanne *et al.* 1987; Wolff-Long *et al.* 1993). However, recombinant myosin heads have not get been functionally expressed in bacteria (McNally *et al.* 1988; Mitchell *et al.* 1986). This may imply bacteria do not contain the required additional proteins for the proper folding of myosin heads. Recombinant heavy meromyosins (HMMs) derived from unconventional myosin V and VI when expressed in insect cells via baculovirus infection, are soluble and functional. Cardiac and skeletal muscle sarcomeric HMMs cannot be produced as functional and soluble proteins in insect cells (Sweeney *et al.* 1994), although HMMs derived from smooth muscle and cytoskeletal type II myosins are also functional when expressed in insect cells (Sweeney *et al.* 1998; Wang *et al.* 2000; Wells *et al.* 1999). This suggests insect cells do not produce additional factors, which are necessary for the folding of cardiac and skeletal muscle myosin heads. The proper folding of the skeletal myosin head has been achieved in C2C12 myogenic cells as skeletal muscle sarcomeric HMMs are soluble and functional when expressed in C2C12 culture (Chow *et al.* 2002; Resnicow *et al.* 2010). These studies indicate that additional proteins are required specifically for proper folding and assembly of muscle sarcomeric myosin heads. Molecular chaperones have been found to play vital roles in the myosin folding and thick filament assembly,

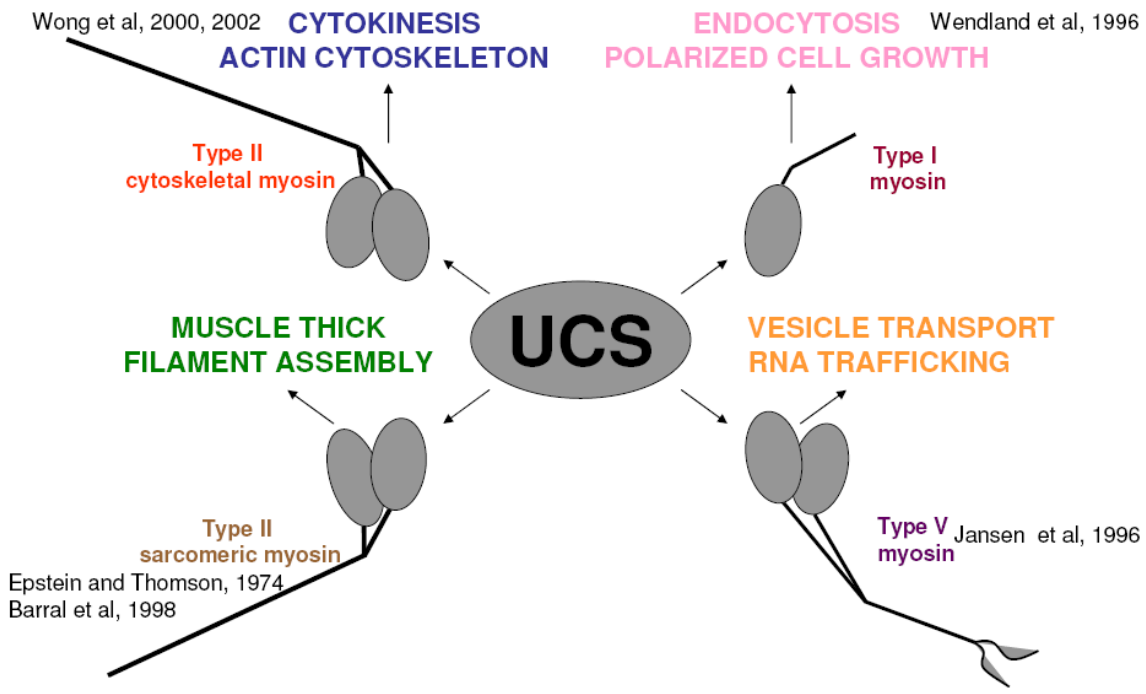
such as UNC-45 (the founding member of UCS domain-containing proteins), heat shock protein 90 (Hsp90) and so on. Mutations of these chaperone proteins lead to impaired myosin organization. The expression level change of these chaperones also affects myosin-related functions.

Molecular Chaperones

Molecular chaperones are proteins that can protect non-native client proteins from misfolding and aggregating and help them achieve their native conformation more easily. Generally, molecular chaperones prevent protein aggregation by irreversibly binding to hydrophobic and/or unstructured regions of the non-native client protein, which are usually buried in the native state (Hartl and Hayer-Hartl 2002). The interaction among the exposed hydrophobic regions of the protein can cause improper intramolecular associations or intermolecular aggregations which inhibit the proper protein folding to the native state. Molecular chaperones are especially essential for proteins to reach their functional structure in cytosol as the aggregation tendency is amplified due to the high local concentration of newly synthesized protein chains on polyribosomes and the effects of macromolecular crowding. Molecular chaperones also serve as quality control for cytoskeletal proteins to prevent defective proteins from impeding their respective assemblies.

UCS Domain-Containing Proteins

UCS domain-containing proteins act as myosin chaperones for many actin- and myosin-dependent cellular processes in many eukaryotic species (Hutagalung *et al.* 2002). UCS domain-containing proteins have been shown to play various important roles in myofibril organization, muscle function, cell differentiation, syncytial-cellular stage transitions, embryonic development, cytokinesis and endocytosis (**Fig. 1.4**). Serious



(modified from Hutagalung *et al.* 2002)

Figure 1.4: Interactions between UCS domain-containing proteins and myosins. The UCS domain-containing proteins can interact with different classes of myosins to produce and maintain different myosin-dependent functions within various organisms (Hutagalung *et al.* 2002).

defects in myosin assembly and consequent cellular processes would occur once genes for UCS-domain containing proteins are absent.

UCS domain-containing proteins have been identified by a conserved C-terminal UCS domain according to sequence homology (Hutagalung *et al.* 2002). The UCS domain is required for almost all myosin-related functions. The name of UCS comes from three founding members (UNC-45 from *C. elegans*, Cro1 from *Podospora anserina*, and She4 from *S. cerevisiae*) (Barral *et al*. 1998). Many experiments have been showing that UCS domain-containing proteins from different organisms are important for the proper folding and subsequent function of myosin heads from a variety of classes of myosins.

UCS domain-containing proteins have been found in fungi, nematodes, insects, fish, amphibians, birds and mammals (Hutagalung *et al.* 2002). The UCS domain-containing proteins can be classified into two large sub-groups: animal UCS proteins and fungal/yeast UCS proteins (**Fig. 1.5**). These two sub-classes share homology only on the C-terminal UCS domain with approximately 50% similarity. The N-terminal region of fungal UCS-containing proteins (some groups call it "central domain") is kind of conserved within some fungi and yeast species. However, the central region or central domain is not well conserved across all species with around 16.5% homology between She4p and the human homologue (Shi and Blobel 2010). The TPR (tetratrico peptide repeat) domain exists only in the animal UCS domain-containing proteins, not in the fungal and yeast homologues.

Animal UCS domain-containing proteins can be further divided into two groups: vertebrate and invertebrate homologues. Invertebrates like *C. elegans* and *Drosophila melanogaster*, contain only one gene for the transcription of a UCS domain-containing protein. However, vertebrates like human beings and mice, have two genes which would

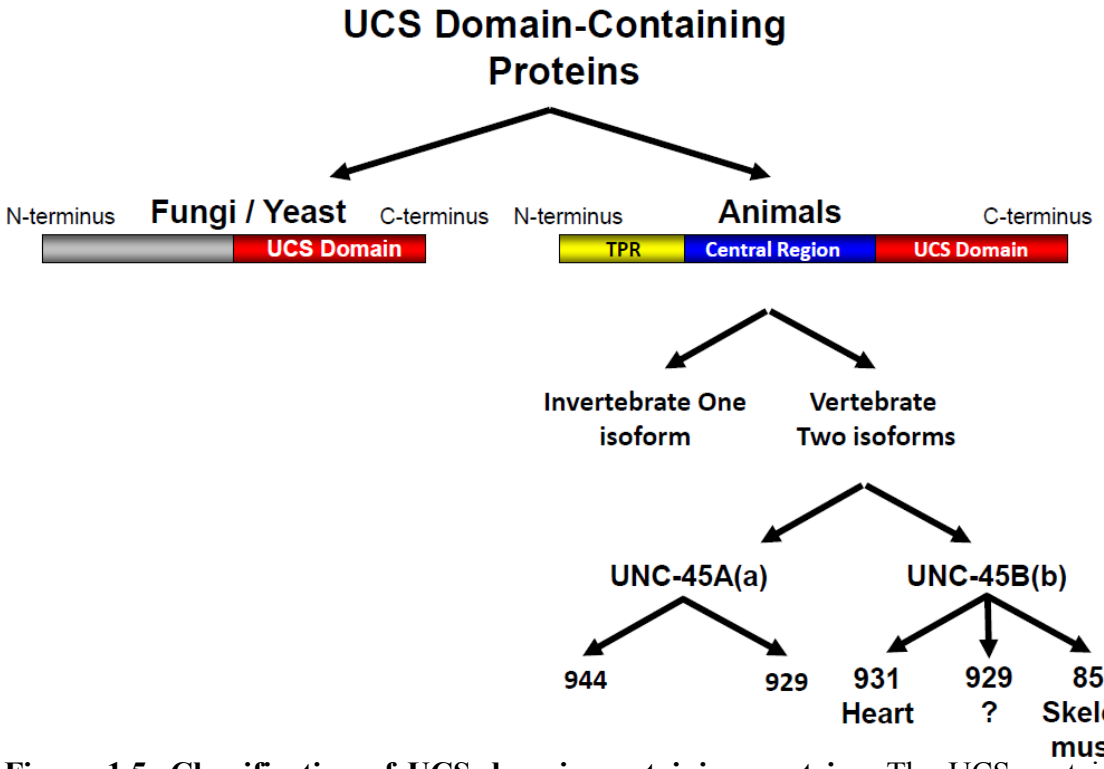


Figure 1.5: Classification of UCS domain-containing proteins. The UCS-containing proteins can be divided into two major categories: fungal and yeast UCS proteins and animal UCS proteins. Animal homologues can be further divided into vertebrate and invertebrate UCS proteins. Vertebrates have two isoforms: UNC-45A(a) and UNC-45B(b). UNC-45A(a) has two spliceoforms and UNC-45B(b) has three spliceforms.

generate UCS domain-containing proteins. UNC-45A(a) is expressed in many human organs, including brain, kidney, liver and so on. UNC-45B(b) is found exclusively in heart and skeletal muscles (Price *et al.* 2002). Brain Allan Stanley's thesis showed that there are three UNC-45B(b) spliceoforms, which may have different distribution among organs. Our lab also showed that there are two UNC-45A(a) spliceoforms, which may have differential functions.

C. elegans UNC-45

C. elegans UNC-45 is one of three founding members for the identification of UCS (UNC-45/CRO1/She4p) domain-containing proteins (Barral *et al.* 1998). As a vertebrate UCS protein, *C. elegans* UNC-45 consists of three regions: the TPR domain, a central region and the UCS domain. Previous studies with recombinant *C. elegans* UNC-45 have found that the N-terminal TPR domain interacts with the C-terminal MEEVD motif of well-known, abundant molecular chaperone Hsp90 *in vitro*, whereas the subfragment containing the central region and the UCS domain interacts with myosin and then exerts the biological activities on myosin heads and the standard chaperone substrate citrate synthase (CS) (**Fig. 1.6;** Barral *et al.* 2002; Hutagalung *et al.* 2002).

The *unc-45* gene was discovered as a recessive temperature-sensitive allele (Epstein and Thomson 1974). Four temperature-sensitive mutations and two lethal mutations within *C. elegans unc-45* have been characterized (Barral *et al.* 1998; Venolia and Waterston 1990). All temperature-sensitive mutations are caused by missense substitutions in the C-terminal part of *C. elegans* UNC-45 (three mutations in the UCS domain and the other one in the central region) and lethal mutations are generated by stop codons within the central region, which result in the premature polypeptide chain termination of the UNC-45 protein (**Fig. 1.6**).

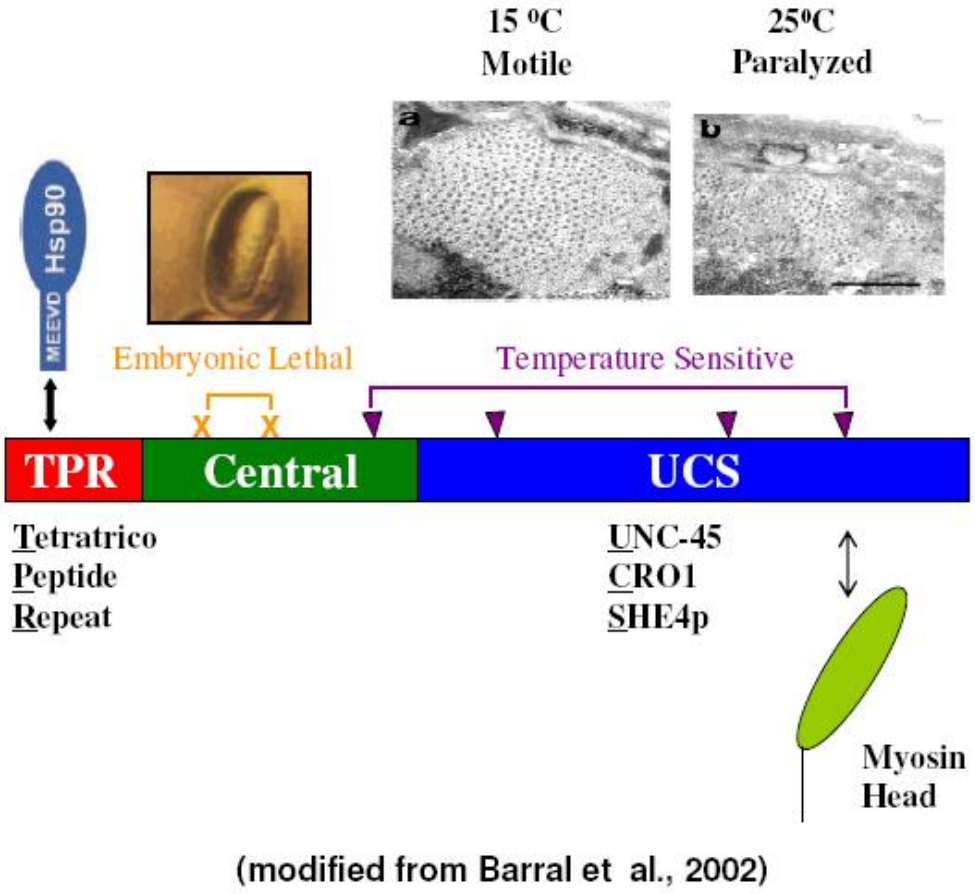


Figure 1.6: Mutations and domain structure of *C. elegans* UNC-45. *C. elegans* UNC-45 has the Hsp90-binding TPR domain on the N-terminus, the central region in the middle with unknown function, and the myosin-binding UCS domain on the C-terminus. Stop-codon mutations in the central region cause embryonic lethality and missense-substitution mutations in the C-terminal part of UNC-45 cause reduced myosin accumulation and thick filament assembly at the restrictive temperature.

The *unc-45* gene has been indicated to be essential during *C. elegans* development through mutations that disrupted myosin-dependent processes. The phenotype of nematodes carrying lethal *unc-45* mutations is similar to that of worms without body-wall muscle myosin heavy chain A: embryonic lethality with arrested development and paralysis at the two-fold stage when no functional body-wall muscle differentiates (**Fig. 1.6**; Venolia and Waterston 1990). All of temperature-sensitive loss-of-function *unc-45* mutants are normal when grown at the permissive temperature (15^0C) but have defective muscle formation and then become paralyzed with marked disorganization of thick filament assembly and decreased myosin accumulation if grown at the restrictive temperature (25^0C) (**Fig. 1.6**; Barral *et al.* 1998). UNC-45 is considered as a chaperone for the folding and assembly of myosin since UNC-45 has the capability of binding to the myosin head and then preventing its thermal aggregation *in vitro* (Barral *et al.* 2002). It is possible that temperature-sensitive mutations within *unc-45* may affect the interaction process between UNC-45 and myosin at the restrictive temperature. These findings suggest an important role for UNC-45 during muscle development.

Previous studies have shown differential UNC-45 localization during nematode development. UNC-45 exists in all worm muscle cells during adulthood. In developing embryos and larvae, UNC-45 is localized in the muscle cell cytosol, but in the adult muscle cells UNC-45 localizes more in the sarcomere (Ao and Pilgrim 2000). *C. elegans* UNC-45 also plays an important role in the organization of the contractile ring during cell division via interaction with non-muscle class II myosins (Kachur *et al.* 2004). Temperature-sensitive mutants also show developmental regulation. Changing the growth temperature from the permissive temperature 15^0C to the restrictive temperature 25^0C for embryos and larvae before sexual maturity leads to decreased body movement, reduced myofilament organization, and scrambled distributions of myosin A and myosin B along

the thick filament. Switching temperatures from the restrictive temperature to the permissive temperature for developing embryos and larvae before adulthood also reverses these mutant phenotypes (Epstein and Thomson 1974). However, huge amount of assembled myosins may not be senstitive to loss-of-function of UNC-45 (Epstein *et al.* 1982; Garcea *et al.* 1978). Therefore, it is indicated that UNC-45 plays an important role in the proper folding of myosin and the appropriate organization of myofilaments during *C. elegans* development.

Recent explorations have revealed that *C. elegans* UNC-45 can be ubiquitinated by an E3/E4 complex and then degraded by the proteasomal system *in vivo* (Hoppe *et al.* 2004). The accumulation of *C. elegans* UNC-45 appears to be strictly regulated and this regulation is important for proper myosin assembly. Our lab confirmed this hypothesis since overexpression of UNC-45 in *suIs2* worms (transgenic wild-type nematodes with integration of UNC-45-overexpressing constructs, driven by *unc-54* promoter for specific body-wall muscle expression) leads to decreased but organized thick filament assembly as well as decreased myosin accumulation (Lansverk *et al.* 2007).

It seems likely that the UCS domain is the most important region of UNC-45 for the myosin binding and assembly because fungal UCS domain-containing proteins do not have the TPR domain and the only conservation among animal UNC-45 proteins and fungal and yeast UCS domain-containing proteins lies within the UCS domain (Hutagalung *et al.* 2002). The capability of binding, chaperoning and preventing aggregation of myosin heads has also been mapped to the UCS-central subfragment of *C. elegans* UNC-45 (Barral *et al.* 2002). In addition, the UCS domain of fungal and yeast UCS protein homologues can rescue their loss-of-function mutants (Lord and Pollard 2004). Therefore, it is highly possible that the UCS domain of *C. elegans* UNC-45 also can rescue *unc-45* loss-of-function mutants.

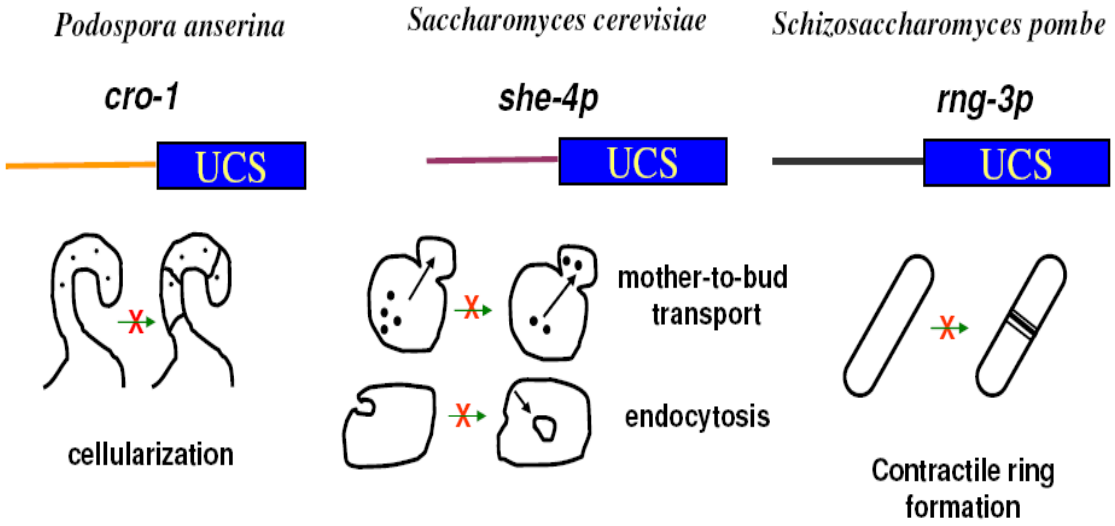


Figure 1.7: Myosin-related defects caused by fungi and yeast UCS protein mutants. In *Podospora anserina*, the absence of Cro-1 leads to the failure of celluraization with polyploidy nuclei. The knockout of She4p in *Saccharomyces cerevisiae* results in the defective endoctyosis and vesicle transport from mother to bud. Missing Rng3p in *Schizosaccharomyces pombe* causes the problem for the contractile ring formation.

Fungal and yeast UCS Proteins

Saccharomyces cerevisiae She4p was identified through two different genetic screens. One screen (the *SHE* screen for Swi5p-dependent HO expression) was looking for expression of the HO endonuclease solely in mother cells but not in buds (Jansen *et al.* 1996). The other screen (the *dim* screen for defective internalization of membrane) was looking for endocytosis defects (Wendland *et al.* 1996). She4p has 789 amino acids with the UCS domain on the C-terminus and one unknown region on the N-terminus. Missing She4p in the budding yeast causes defects in endocytosis, altered cytoskeleton, and defective mRNA transport to bud (**Fig. 1.7**). The mutation of She4p also results in decreased growth, defective secretion, and constitutive rounding of cells. She4p can interact with the motor domains of myosin classes V and I through the conserved UCS domain. No functional class I myosin can be produced in *sheΔ Saccharomyces cerevisiae* as its motor domain is defective in actin binding.

Podospora anserina Cro1 was discovered in a screen for sexual sporulation failure (Berteaux-Lecellier *et al.* 1998). Cro1 has 702 amino acids. Similar to She4p, Cro1 also contains the C-terminal UCS domain and one unknown N-terminal region. Cro1 is roughly 21% identical and 40% similar compared to She4p. Missing Cro1 leads to abortive meiosis, inability to organize septa between the daughter nuclei after mitotic division, and reduced filamentous growth (**Fig. 1.7**). In the *cro1* null mutant, the actin cytoskeleton also becomes disordered and myosins can not function to interact with the cytoskeleton.

Schizosaccharomyces pombe Rng3p is important for the formation of myosin containing progenitor spots. Rng3p mutant cells do not produce myosin containing progenitor spots and fail to have cytokinesis, leading to the generation of spores with multiple nuclei (Wong *et al.* 2000). Temperature sensitive Rng3p mutant cells also fail to

maintain the actomyosin ring when shifted from the permissive temperature to the restrictive temperature. Swo1p, the Hsp90 homologue in *S. pombe*, has been indicated to be involved in proper organization of the actomyosin ring. Temperature sensitive Swo1p mutants have problems during cell division and actomyosin ring organization (Munoz and Jimenez 1999). However, only Rng3p not Hsp90 is required to restore *in vitro* actin-activated ATPase and motility activity of recombinant *S. pombe* class II myosins (Lord and Pollard 2004). It is hypothesized that Swo1p is necessary for organization of the actomyosin ring and Rng3p is also essential for stabilization of the actomyosin ring after it is formed.

Chapter 2: Materials and Methods

Excerpts with permission from:

The Myosin-Binding UCS domain but not the Hsp90-Binding TPR domain of the UNC-45 Chaperone is Essential for Function in *Caenorhabditis elegans*.

(Weiming Ni, Alex H. Hutagalung, Shumin Li and Henry F. Epstein (2011). *J. Cell. Sci.* 124, 3164-3173)

General *C. elegans* Methods

N2 (wild-type), CB286 (*e286*) and LV15 (*st601*) were obtained from the CGC (Caenorhabditis Genetics Center). *suIs2* (integrated lines overexpressing UNC-45) worms were as described previously (Landsverk *et al.* 2007). N2, CB286 and LV15 were grown on NGM plates for analysis of phenotypes (Brenner 1974; Epstein and Thomson 1974; Venolia and Waterston 1990). *suIs2* worms were grown on 8P plates for biochemical studies (Sulston and Brenner 1974). Transgenic worms with body-wall muscle specific extrachromosomal arrays of the P_{unc-54}::unc-45 fragment^{FLAG} were generated by microinjection as previously described (Hoppe *et al.* 2004; Mello and Fire 1995; Stinchcomb *et al.* 1985).

Transgene Construction

The cDNA sequence of *C. elegans unc-45* fragments with a 3' FLAG tag sequence were subcloned into pPD30.38 (Andrew Fire Lab, Stanford University, CA).

(1) FL contains all 961 amino acids, amplified with primers as follows.

Forward: 5'-CGGGGTACCCCGATGGTTGCTCGAGTACAGACT-3'

Reverse: 5'-CGGGGTACCCCGTCACTTGTCATCGTCGTCCTTGTAGTCGG ATCCTTCCTGAATGGTGCTCAT-3'

(2) TPR(-) is amino acids 135 to 961, cloned by following primers.

Forward: 5'-CGGGGTACCCCGATGACCACTTCACTGGCTAAT-3'

Reverse: 5'-CGGGGTACCCCGTCACTTGTCATCGTCGTCCTTGTAGTCGG ATCCTTCCTGAATGGTGCTCAT-3'

(3) UCS is amplified from amino acids 524 to 961 with primers as follows.

Forward: 5'-CGGGGTACCCCGATGGCAGTGATCAGTTTGGCG-3'

Reverse: 5'-CGGGGTACCCCGTCACTTGTCATCGTCGTCCTTGTAGTCGG ATCCTTCCTGAATGGTGCTCAT-3'

(4) UCS(-) is cloned from amino acids 1 to 523 using following primers.

Forward: 5'-CGGGGTACCCCGATGGTTGCTCGAGTACAGACT-3'

Reverse: 5'-CGGGGTACCCCGTCACTTGTCATCGTCGTCCTTGTAGTCGG ATCCTTCTTCTTTCATCGTTGC-3'

Immunoblotting

Young-adult worms were hand-picked, placed in SDS sample buffer, heated at 95°C for 10 min, and after brief cooling, run in 7.5% SDS-PAGE to minimize protein degradation (Miller *et al.* 1983; Zengel and Epstein 1980). Mouse monoclonal anti-FLAG M2-peroxidase (HRP) antibody (Sigma-Aldrich), Hsp90 mouse monoclonal antibody AC88 (Stressgen), rabbit polyclonal anti-*C. elegans* UNC-45 antibody (gift of Dr. Thorsten Hoppe, University of Cologne, Germany), mouse monoclonal anti-*C. elegans* MHC A (myosin heavy chain A) antibody (mAb 5-6), mouse monoclonal anti-*C. elegans* MHC B (myosin heavy chain B) antibody (mAb 28.2), and mouse monoclonal anti-*C. elegans* MHC D (myosin heavy chain D) antibody (mAb 5-17) (Miller *et al.* 1983) were employed for specific immunoblotting (Barral *et al.* 1998; Landsverk *et al.* 2007). Densitometry of the exposed films was performed using AlphaEaseFC software (Alpha Innotech). A. U. means arbitrary unit.

Microscopy

Immunofluorescence microscopy on whole mounts of young-adult nematodes was performed according to Finney and Ruvkun (1990). Worms were reacted with FITC-conjugated mAb 5-6 (mouse monoclonal anti-*C. elegans* MHC A (myosin heavy chain A) antibody) (Miller *et al.* 1983) and imaged using an Axioplan 2 microscope with a 40×0.75 plan-NEOFLUAR objective, equipped with an AxioCam MRc5 digital camera, and processed through AxioVision 3.0 software (Carl Zeiss MicroImaging, Inc.) at room

temperature (Barral *et al.* 1998; Landsverk *et al.* 2007). The number of A bands per body-wall muscle cell in region III of transgenic worms was counted.

Motility Assays

Individual young-adult nematodes were placed in M9 buffer, and body bends were counted for 15 second intervals (Epstein and Thomson 1974).

Pull-down Assays

In Fig. 4.10A, *suIs2* worms were lysed and incubated with anti-FLAG beads (Sigma-Aldrich) for 2 h at 4°C. After washing beads with lysis buffer three times, proteins were eluted by FLAG peptide (Sigma-Aldrich) three times and run in a 7.5% SDS-PAGE for immunoblotting (Barral *et al.* 2002). In Fig. 4.10B, *C. elegans* myosins from worm lysate were immobilized on beads coupled to mouse monoclonal anti-*C. elegans* MHC B (myosin heavy chain B) antibody (mAb 28.2) (Miller *et al.* 1983). 1.8 μM recombinant *C. elegans* Hsp90 purified from *E. coli* and different amounts of recombinant *C. elegans* UNC-45 purified from Sf9 insect cells were then added to the fixed concentration of myosin beads for interaction. After washing, the resulting protein complexes were analyzed by a 7.5% SDS-PAGE, Coomassie blue staining and densitometry using a BioRad Gel Doc 2000 system with Quantity One Quantitation Software (Bio-Rad) (Barral *et al.* 2002). In Fig. 4.9, different amounts of purified recombinant *C. elegans* UNC-45 or Hsp90 were added to the fixed concentration of myosin beads for interaction. Apparent 50% binding of Hsp90 or UNC-45 to myosin was estimated by SigmaPlot Software (Systat Software, Inc.).

Statistics

The two-tailed Student's *t* test was used to evaluate the statistical significance of the result at the 95% confidence level; a *P* value less than 0.05 was considered to indicate statistical significance.

Chapter 3: The Myosin-Binding UCS Domain Possesses Intrinsic Biological Activity *in vivo*

Adapted with permission from:

The Myosin-Binding UCS domain but not the Hsp90-Binding TPR domain of the UNC-45 Chaperone is Essential for Function in *Caenorhabditis elegans*.

(Weiming Ni, Alex H. Hutagalung, Shumin Li and Henry F. Epstein (2011). *J. Cell. Sci.* 124, 3164-3173)

INTRODUCTION

Several studies indicate that proper myosin folding and assembly require the function of additional proteins. Recombinant myosin motor domains cannot be expressed in bacteria to produce soluble and functional proteins (McNally *et al.* 1988) but, when expressed in cultured C2C12 myogenic cells, are functional (Chow *et al.* 2002; Resnicow *et al.* 2010). The chaperones UNC-45 and heat shock protein 90 (Hsp90) have been implicated in proper myosin folding and thick filament assembly (Du *et al.* 2008). Chaperone deficiency or chaperone excess can lead to decreased myosin assembly and accumulation in *C. elegans* as lower or overexpression of UNC-45 in *C. elegans* results in defective myofibril organization (Barral *et al.* 1998; Landsverk *et al.* 2007; Fig. 3.1).

UNC-45 contains three regions: an N-terminal tetratricopeptide repeat (TPR) domain, a central region, and a C-terminal UCS domain (Barral *et al.* 1998). The TPR domain has been shown to interact with Hsp90 (Barral *et al.* 2002; Russell *et al.* 1999; Scheufler *et al.* 2000). The function of the central region has not been determined as yet in *C. elegans*. It has been reported that the central region mediates Z line association and interacts with Apo2a (the cytidine deaminase Apobec2a) in zebrafish (Etard *et al.* 2008, 2010). The UCS domain is named for three early identified proteins (UNC-45 from *C. elegans*, Cro1 from *Podospora anserina*, and She4 from *S. cerevisiae*) and interacts with myosin motor domains (Barral *et al.*1998, 2002; Lord and Pollard 2004; Toi *et al.* 2003). The yeast and fungal homologs show similarity to UNC-45 only in the carboxyl terminal UCS domains.

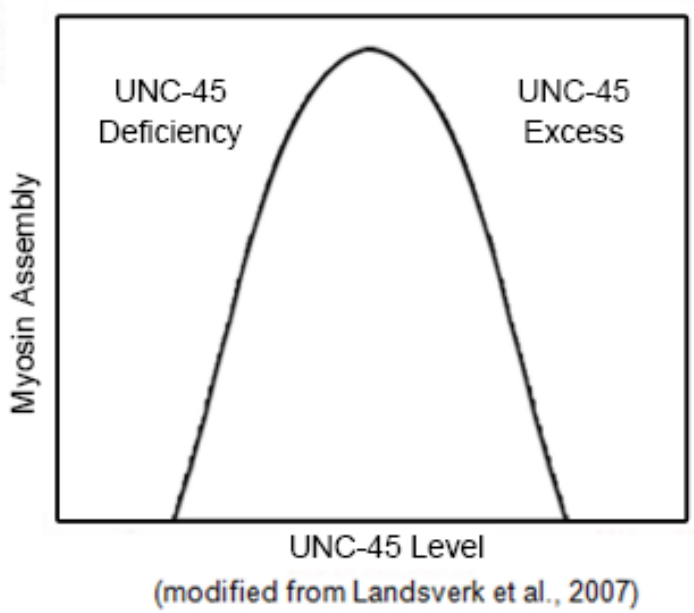


Figure 3.1: Model of myosin assembly dependence upon UNC-45 protein levels in *C. elegans* body-wall muscle cells. Myosin heavy chain accumulation and its consequent assembly are controlled by protein degradation on either side of the optimal UNC-45 concentration range (Landsverk *et al.* 2007).

UNC-45 orthologs from *C. elegans* to humans may function as putative Hsp90 co-chaperones (Barral *et al.* 1998, 2002; Price *et al.* 2002; Young *et al.* 2003). *In vitro* myosin folding experiments suggest Hsp90-dependent folding of the myosin motor domain is activated by the muscle-specific isoform UNC-45b because this activation is blocked by the Hsp90 inhibitor geldanamycin (Liu *et al.* 2008). Furthermore, both UNC-45b and Hsp90 colocalize in cytoplasmic complexes with sarcomeric myosin during myofibril assembly in C2C12 myogenic cultures (Liu *et al.* 2008; Mishra *et al.* 2005; Srikakulam *et al.* 2008). These results suggest that UNC-45 may serve to modulate Hsp90 function.

Multiple studies indicate that certain functions of UNC-45 and its fungal UCS-domain homologs may be independent of Hsp90 *in vivo* and *in vitro* (Toi *et al.* 2003). The purified recombinant Rng3 UCS domain alone completely restored *in vitro* motility of pure Myo2, the myosin-II motor of the fission yeast *S. pombe* (Lord and Pollard 2004). Recombinant expression of yeast UCS proteins or their UCS domains alone can rescue loss-of-function temperature-sensitive mutants of She4p in the budding yeast *S. cerevisiae* and Rng3p in the fission yeast *S. pombe* (Lord and Pollard 2004; Lord *et al.* 2008). In zebrafish, deletion of the N-terminal TPR domain has no effect on the disruptive activity of UNC-45b on myosin thick filament organization, whereas deletion of the C-terminal UCS domain abolishes the disruptive effect of UNC-45b overexpression (Bernick *et al.* 2010). UNC-45b and Apo2 proteins act in a Hsp90 independent pathway that is required for integrity of the myoseptum and myofiber attachment (Etard *et al.* 2010). Alternatively, Hsp90 may also function in inhibitory as well as activating functions with respect to UNC-45.

In this chapter, I test whether the UCS domain of UNC-45 exhibits intrinsic biological activity as full-length protein in *C. elegans* body-wall muscle *in vivo*. I cloned various UNC-45 constructs for generation of transgenic worms: FL contains all three regions; TPR(-) consists of the central region and UCS domain which cannot bind Hsp90; UCS is the UCS domain only that can bind myosin; UCS(-) is composed of the Hsp90-binding TPR domain and central region which cannot bind myosin motor domains (Barral *et al.* 2002; Fig 3.2). The relationship between UNC-45 levels and myosin assembly in Fig. 3.1, based upon our earlier work, serves as the basis for the experimental design of this project (Landsverk *et al.* 2007). Overexpression of transgenic UNC-45 and its fragments in wild-type genetic background was examined. The UCS domain has been shown to possess intrinsic chaperone activity as full-length UNC-45 in wild-type *C. elegans* body-wall muscle.

RESULTS

Transgenic Expression of the Myosin-Binding UCS Domain Shows the Greatest Reduction of Wild-Type Nematode Motility

The overexpression of UNC-45 in the N2 wild-type background results in decreased motility and reduced A-band assembly (the assemblage of myosin-containing thick filaments) (Landsverk *et al.* 2007; Fig.3.1). To investigate the consequences of expressing different UNC-45 fragments in wild-type worms, I generated specific transgenic lines in N2 by microinjection. These worms carried extrachromosomal arrays to express different UNC-45 fragments under control of the strong *unc-54* promoter that localizes expression to body-wall muscles (Fig. 3.2).

Figure 3.2: Regional sequences of *C. elegans* UNC-45 constructs. Fragments cloned for injection under control of the body-wall muscle specific *unc-54* (myosin heavy chain B) promoter with the FLAG tag on the C-terminus are listed. The *unc-54* promoter results in high output of UNC-45 fragments in comparison to the low output *unc-45* promoter.

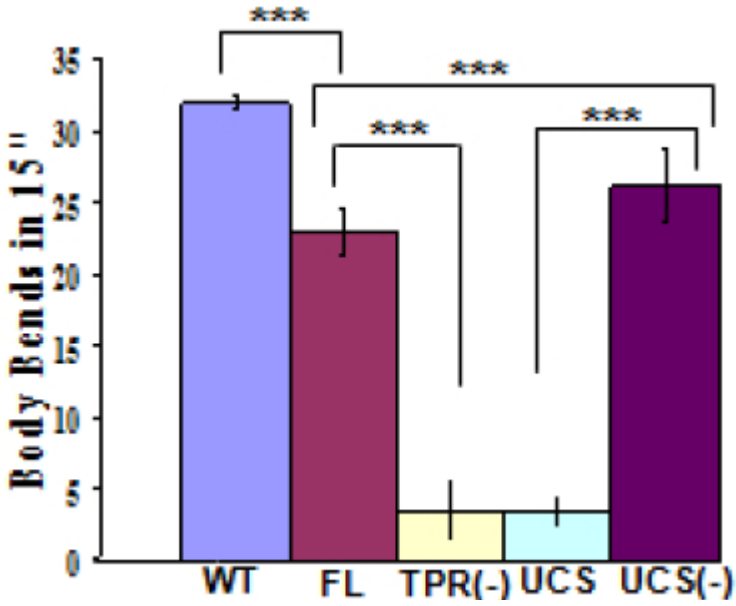


Figure 3.3: Reduction of wild-type nematode motility by transgenic UCS-containing proteins. The UCS domain and TPR(-) construct significantly decrease wild-type nematode motility compared to full-length UNC-45 and UCS(-) fragment. Error bars indicate mean ± s.d. ***P<0.001.

I found that FL, TPR(-), and UCS, but not UCS(-), were able to markedly decrease the motility of wild-type worms (Fig. 3.3). The expression of FL as a positive control decreased the motility of wild-type as previously described (Landsverk *et al.* 2007). TPR(-) and UCS were able to further decrease the motility of wild-type. UCS(-) expression affected the motility of wild-type worms less than other UNC-45 fragments. The UCS domain and TPR(-) fragments, which can bind myosin but not Hsp90, were most active in decreasing the motility of wild-type worms. Expression of UCS(-), which can bind Hsp90 but not myosin, clearly affected motility less than the other UCS-containing proteins (Fig. 3.3).

Transgenic Expression of the Myosin-Binding UCS Domain Shows the Greatest Reduction of Wild-Type Nematode A-Band Assembly

Sarcomere assembly in transgenic wild-type worms was investigated by labeling A bands with FITC-conjugated monoclonal antibody (mAb) 5-6 (anti-MHC A). The reduction of A-band assembly was detected in transgenic FL, TPR(-) and UCS but not in the UCS(-) or control parental wild-type lines (Fig. 3.4). Wild-type worms had normal thick filament assembly. The FL protein was able to reduce A-band assembly. The TPR(-) and UCS fragments were able to further reduce A-band assembly. In contrast, the UCS(-) fragment did not appear to reduce thick filament assembly. In summary, the UCS domain and TPR(-) fragment reduce A-band assembly significantly more than either FL or UCS(-) in wild-type.

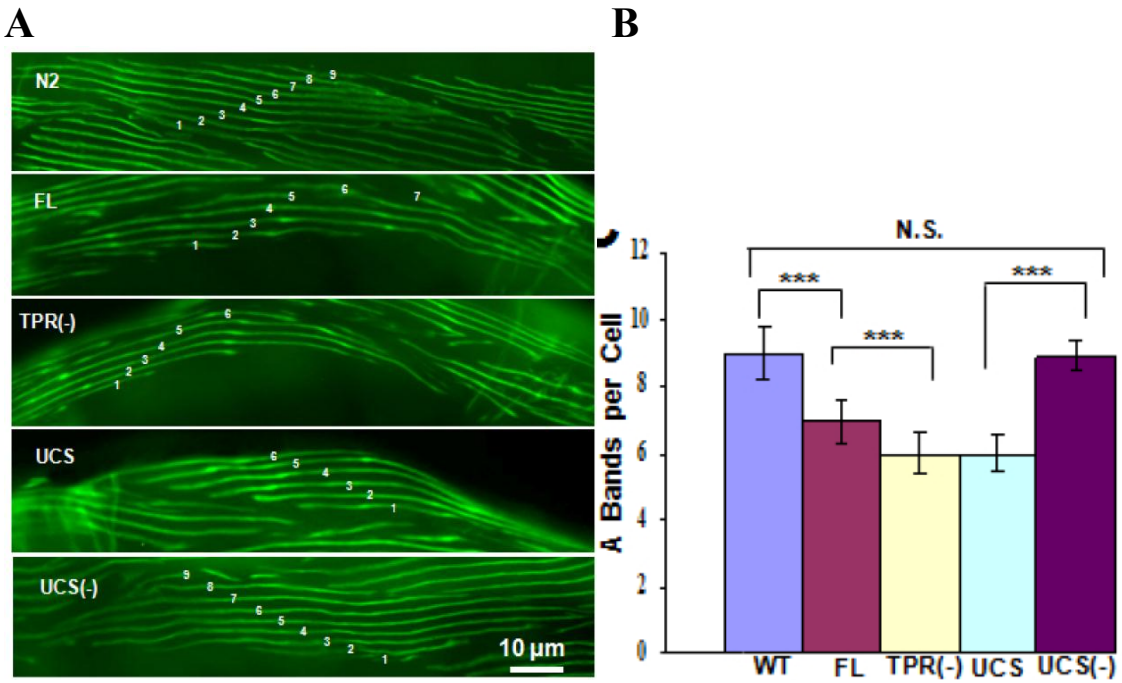


Figure 3.4: The UCS domain and TPR(-) construct significantly diminish A-band assembly in wild-type nematodes. (A) Myosin heavy chain A staining shows different A-band assembly in transgenic wild-type worms expressing distinct UNC-45 fragments. (B) Quantification of A-band assembly diminution of transgenic wild-type worms expressing UNC-45 fragments. Error bars indicate mean ± s.d. *** P<0.001. N.S., not significant.

Transgenic Expression of the Myosin-Binding UCS Domain Shows the Greatest Reduction of Wild-Type Nematode Body-Wall Muscle Myosin Accumulation

The different extent of reduction of motility and myosin assembly in transgenic wild-type worms expressing various UNC-45 fragments could be due to distinct levels of expression of the fragments or differences in their intrinsic activities. The accumulation of these transgenically expressed fragments in wild-type worms was verified by immunoblots with the anti-FLAG antibody. The accumulation of the UCS fragment in wild-type was discovered to be significantly lower (almost undetectable) than that of the other transgenic products (Fig. 3.5A). Body-wall muscle specific myosins A and B were determined by immunoblots at the same time. Pharyngeal myosin D was used as loading control. Densitometric ratios of body-wall myosins A and B compared to myosin D show that UCS and TPR(-), but not UCS(-), reduced myosin accumulation similarly to FL (Fig. 3.5A). Using rabbit polyclonal anti-*C. elegans* UNC-45 antibody (gift of Dr. Thorsten Hoppe, University of Cologne, Germany) that reacts with all three UNC-45 regions, UCS protein was confirmed to be expressed in lower amounts (Fig. 3.5B). Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot. These results demonstrate that the UCS domain, but not the TPR domain, modifies myosin accumulation and its consequent assembly in this assay.

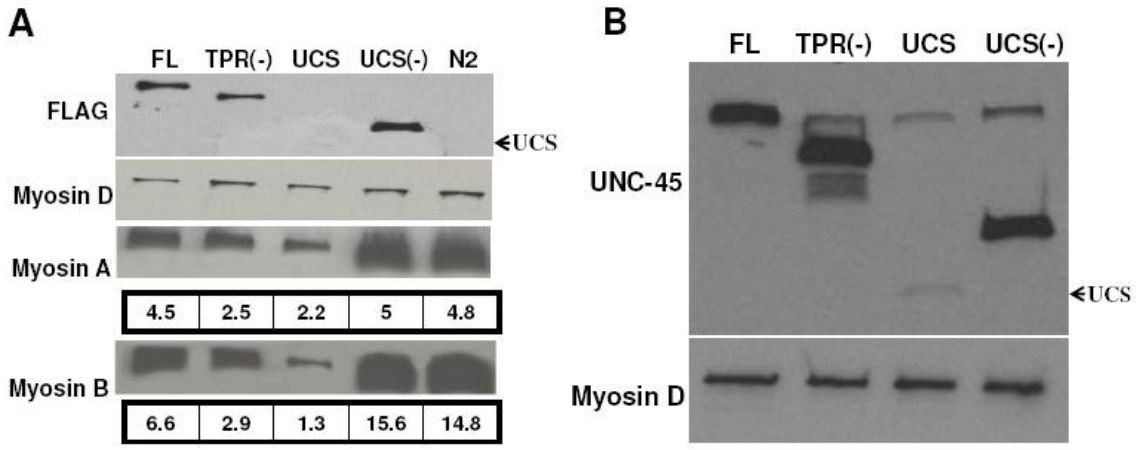


Figure 3.5: The UCS domain is expressed at a lower level compared to other transgenic proteins and the myosin-binding truncates show reduction of body-wall muscle myosin accumulation similar to FL UNC-45 in wild-type nematodes. (A) Transgenic expression of UNC-45 fragments was detected by immunoblots with anti-FLAG antibody. UCS was almost invisible by anti-FLAG antibody. Body-wall muscle specific myosin heavy chains A and B were detected and quantified by immunoblots with mAb 5-6 and mAb 28.2. Pharyngeal myosin heavy chain D, detected by mAb 5-17, was used as loading control. (B) UNC-45 truncate expressions in wild-type were examined by immunoblots with rabbit polyclonal anti-*C. elegans* UNC-45 antibody. UCS and other fragments were detectable on the blots. Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot. Pharyngeal myosin heavy chain D was used as loading control.

Quantification of the Transgenic Expression of the Myosin-Binding UCS Domain in Wild-type Nematodes

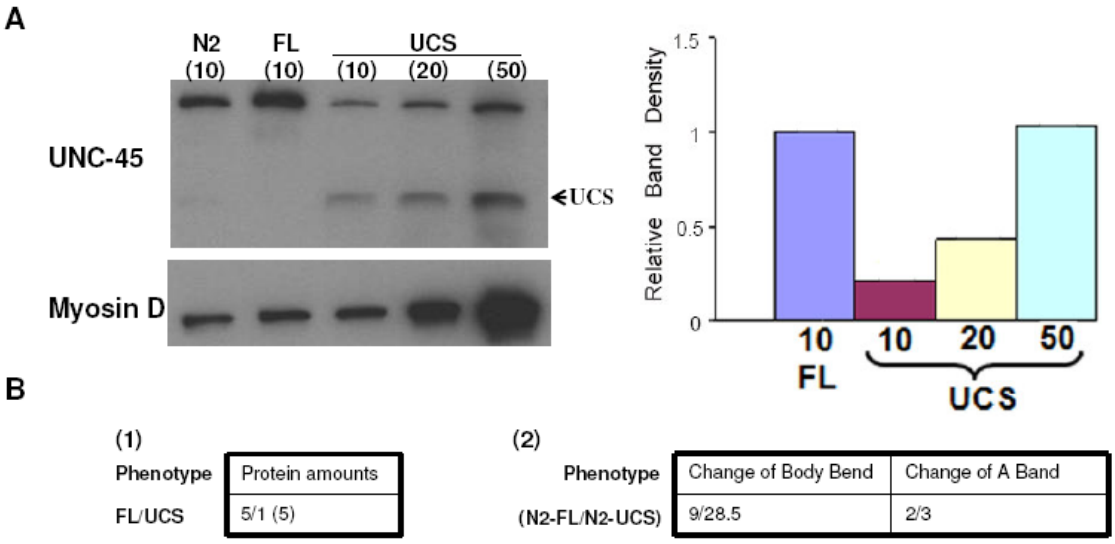
The number of transgenic UCS worms in wild-type per lane on immunoblots was varied in order to semi-quantitatively compare UCS expression levels relative to FL. Rabbit polyclonal anti-*C. elegans* UNC-45 antibody was used to enhance the detection of the UCS fragment (Fig 3.6A). Quantification of the immunoreacted proteins showed that the UCS fragment was expressed at about one fifth of the FL protein level. With this quantification, ratios of FL versus UCS activities were calculated (Fig. 3.6B). UCS was strikingly more active on a per molecule basis in reducing A-band assembly and motility of myosin in wild-type worms than full-length protein.

DISCUSSION

Previous studies have demonstrated that UNC-45 functions as a myosin chaperone that regulates myosin accumulation and assembly by linkage to the ubiquitin proteasome system for degradation (Landsverk *et al.* 2007). In this chapter, I have shown that the UCS domain exhibits greater chaperone activity for myosin accumulation and assembly *in vivo* compared to full-length UNC-45 protein.

In the transgenic expression of full-length and fragments of UNC-45 in a wild-type background, UCS and TPR(-) led to decreased worm motility, A-band assembly and body-wall muscle myosin accumulation as did full-length UNC-45 (Figs. 3.3, 3.4, 3.5). Low levels of UCS could drastically alter the wild-type phenotype compared to FL (Figs. 3.5, 3.6). The wild-type experiment with the UCS(-) fragment further verified that without the highly conserved UCS domain, the TPR domain plus central region show the least reductions. Full-length UNC-45 can bind the well-known molecular chaperone Hsp90 through its TPR domain whereas the UCS domain can bind myosin but not Hsp90. Therefore, this result could be explained by three possibilities: dominant-negative effects

of the UCS domain, the inhibition of the UCS domain's function by the interaction between Hsp90 and the TPR domain, and the inhibition of the UCS domain's activity due to the existence of the TPR domain itself. In order to clarify the effects of the UCS domain on myosin, mutant worms overexpressing these UNC-45 fragments would be analyzed in the Chapter 4.



B

(1)

Phenotype	Protein amounts
FL/UCS	5/1 (5)

(2)

Phenotype	Change of Body Bend	Change of A Band
(N2-FL/N2-UCS)	9/28.5	2/3

Figure 3.6: Quantification of the transgenic expression of the UCS domain in wild-type nematodes. (A) A titration experiment of transgenic UCS worms in wild-type was performed to semi-quantitatively compare the level of UCS expression to that of FL in FL transgenic wild-type by rabbit polyclonal anti-*C. elegans* UNC-45 antibody. Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot. UCS became detectable by polyclonal anti-*C. elegans* UNC-45 antibody. Pharyngeal myosin heavy chain D was used as loading control. (B) Table (1) shows the ratio of protein amounts. Table (2) shows ratios of the decreased body bends and the diminished A bands between transgenic FL and UCS worms, compared to wild-type.

Chapter 4: The Interaction between the TPR Domain and Hsp90 Inhibits the Function of the UCS Domain on Myosin Assembly in *C. elegans* Body-Wall Muscle

Adapted with permission from:

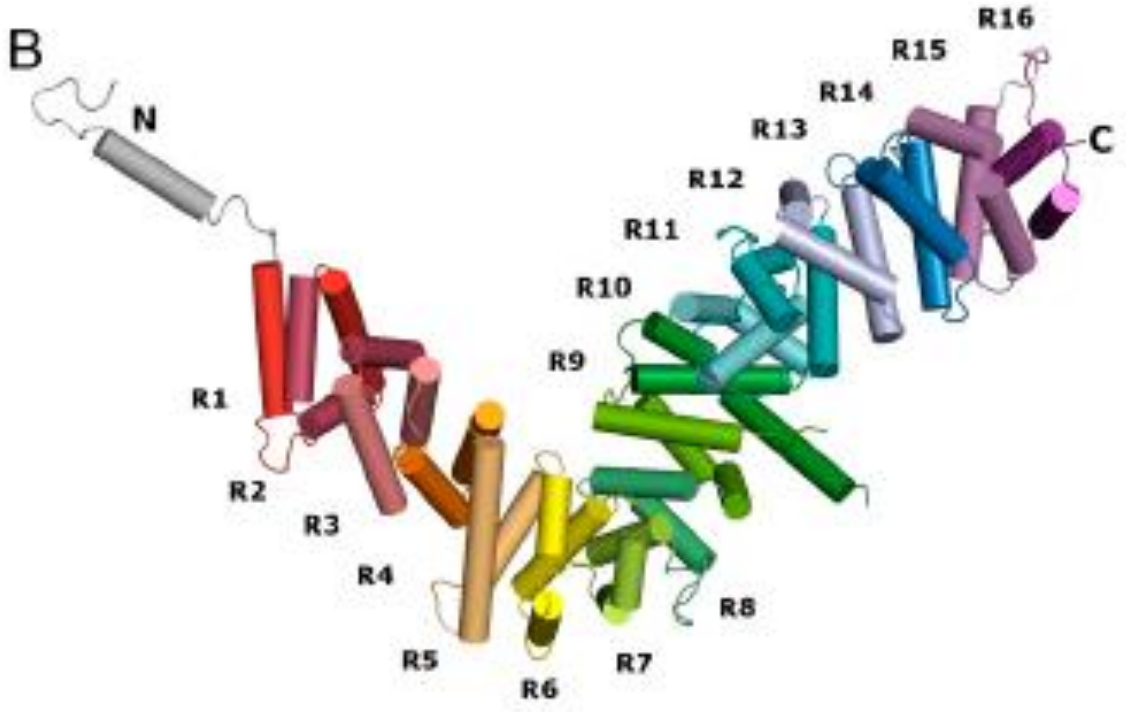
The Myosin-Binding UCS domain but not the Hsp90-Binding TPR domain of the UNC-45 Chaperone is Essential for Function in *Caenorhabditis elegans*.

(Weiming Ni, Alex H. Hutagalung, Shumin Li and Henry F. Epstein (2011). *J. Cell. Sci.* 124, 3164-3173)

INTRODUCTION

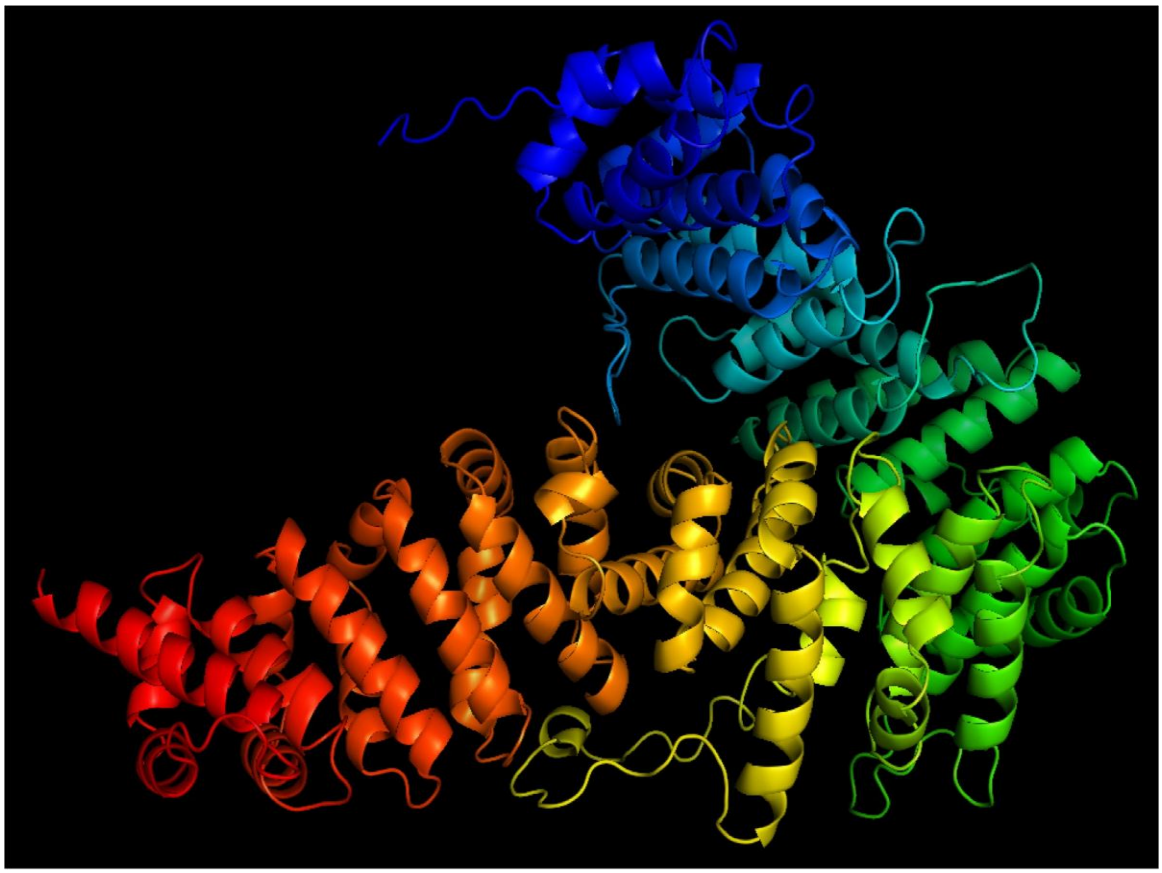
The structure of She4p from *S. cerevisiae* has been determined by X-ray crystallography (Shi and Blobel 2010). They proposed that She4p is a dimer of L-shaped molecules (Fig. 4.1). As a yeast UNC-45 homolog, She4p does not contain the TPR domain on the N-terminus.

C. elegans is monomeric in solution (Barral *et al.* 2002). *Drosophila* UNC-45 is also a monomer in solution (Lee *et al.* 2011). The X-ray crystal structure of *Drosophila* UNC-45 has recently been published (Lee *et al.* 2011). *Drosophila* UNC-45 is also proposed as a monomer with a contiguous series of stacked armadillo repeats in the crystal (Fig. 4.2). Although the TPR domain is disordered in the crystal, the structure of *Drosophila* UNC-45 is quite similar to L-shaped She4p with steric constraints between the N-terminus and C-terminus of the molecule. This result is more relevant to my work because the worm and fly proteins show about 40% identity across all three regions of UNC-45 whereas She4p or Rng3p from *S. pombe*, share more limited identity only in the carboxyl UCS but not the amino terminal domains. Although the *S. cerevisiae* UCS protein She4p in the crystal led to dimerization through multiple alanine substitutions in the N-terminal helix, wild-type She4p was still more than 90% monomeric in solution (Shi and Blobel 2010). Mouse UNC-45 proteins are also monomeric in solution (Liu *et al.* 2008; Srikakulam *et al.* 2008). These proteins (UNC-45a and b) are also more closely related to *C. elegans* UNC-45 than the fungal proteins.



(from Shi and Blobel 2010 with permission)

Figure 4.1: She4p is L-shaped. She4p is composed of 16 helical repeats. These 16 helical repeats are demonstrated in different colors and are numbered from R1 to R16. 16 repeats of She4p are compacted into L-shaped superhelix. The N-terminal loop-helix-loop motif is colored in silver and the N-terminal R16 is colored in purple.



(modified from Lee *et al.* 2011)

Figure 4.2: *Drosophila* UNC-45 assumes a similar L-shape structure. The central region and the UCS domain of *Drosophila* UNC-45 consist of a contiguous series of stacked armadillo repeats. The C-terminus of *Drosophila* UNC-45 is colored in red and the N-terminus of *Drosophila* UNC-45 is colored in blue. There are 17 consecutive helical layers, which are compacted into five discrete armadillo repeats.

The hypothesis that Hsp90 activates the UNC-45b muscle isoform is based upon several correlative lines of evidence. That the two proteins co-localize in C2C12 mouse myogenic cells (Liu *et al.* 2008; Srikakulam *et al.* 2008) does not necessarily imply that they physically interact and could be consistent with either an activating or inhibiting role for Hsp90. The effects of the Hsp90 inhibitor geldanamycin upon myofibril assembly in C2C12 cells could be the result of any one of a number of interactions within the myoblasts, dependent or independent upon UNC-45. The interactome of Hsp90 in a variety of cell types is quite large (Falsone *et al.* 2005; Zhao *et al.* 2005; Zhao and Houry 2007). To my knowledge, there is no physical evidence for the existence of a ternary complex for UNC-45, Hsp90 and myosin.

Chadli et al. (2006) show that UNC-45A may inhibit the Hsp90-dependent maturation of the human progesterone receptor. Hsp90 has also been reported to inactivate glucocorticoid and estrogen receptors by blocking the access to their DNA binding domains (Eilers *et al.* 1989; Picard *et al.* 1988). Moreover, Hsp90 can inhibit the activation of promoter-dependent transcription by glucocorticoid receptors (Kang *et al.* 1999). Hsp90 is also involved in the disruption of transcriptional regulatory complexes (Freeman and Yamamoto 2002). Therefore, Hsp90 is not only necessary for the folding and assembly of active client proteins, but may also be important for the inactivation of client proteins.

In this chapter, I examine the effects of the interactions of Hsp90 and UNC-45 in *C. elegans* body-wall muscle *in vivo* and with *C. elegans* proteins *in vitro*. Various UNC-45 constructs cloned for generation of transgenic worms were as shown in Fig. 3.2. The relationship between UNC-45 levels and myosin assembly in Fig. 3.1 serves as the basis for the experimental design of this project (Landsverk *et al.* 2007). Rescue of loss-of-function mutants by transgenic UNC-45 and its fragments were tested. The *st601 pat*

(paralyzed at two-fold) stop-codon mutation in the central region prevents egg development around the two-fold stage, and the amino acid substitution mutation *e286* in the UCS domain results in severely paralyzed worms with pronounced disorganization of the sarcomere at the 25 ^{o}C restrictive temperature (Epstein and Thomson 1974; Venolia and Waterston 1990; Fig. 4.3). My *in vivo* and *in vitro* results suggest a novel inhibitory role of Hsp90 with respect to UNC-45 in *C. elegans* body-wall muscle.

RESULTS

The Myosin-Interacting UCS Domain, but not the Hsp90-Binding TPR Domain, is Necessary for Rescue of the Embryonic Lethal *st601* Mutant and the Temperature-Sensitive UNC *e286* Mutant

To test the possible dominant-negative effects of TPR(-) and UCS fragments on myosin, I studied the effects of expression of these fragments by rescue of *unc-45* loss-of-function mutants *in vivo*. In many cases, particularly in the body-wall muscle cells of *C. elegans*, expression of transgenes is mosaic (Fire 1986; Fire and Waterston 1989; Krause *et al.* 1994; Okkema *et al.* 1993). This condition results from the fact that body-wall muscle cells arise from multiple embryonic cell lineages, with the number of cell divisions from zygote to terminally differentiated muscle cells varying from 7 to 13 (Sulston *et al.* 1983). The opportunity for losing or silencing extrachromosomal constructs or even integrated transgenes exists (Landsverk *et al.* 2007). Partial rescue is commonly the level of rescue in transgenic *C. elegans* nematodes. Therefore, the term "rescue" should be taken to mean "partial rescue" as the result of mosaicism.

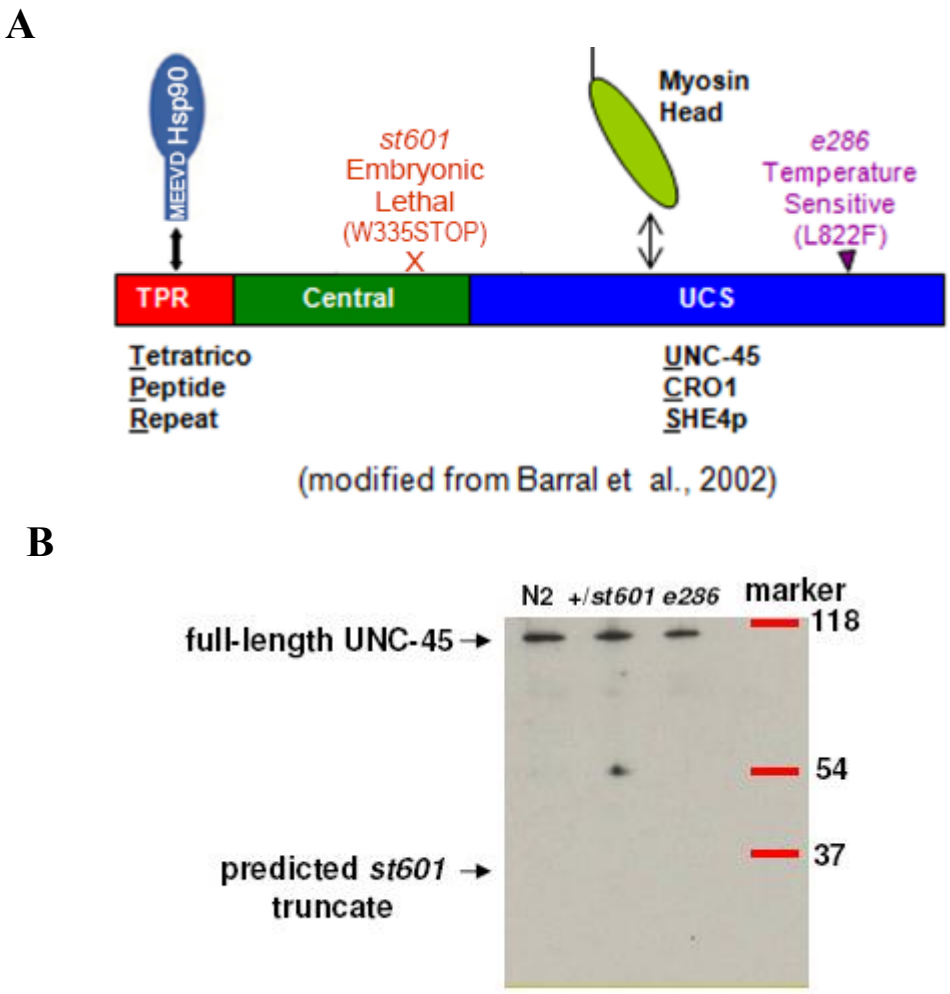


Figure 4.3: *C. elegans* UNC-45 mutations. (A) UNC-45 contains the N-terminal Hsp90-binding TPR domain, the central region, and the C-terminal myosin-binding UCS domain (Barral *et al.* 1998, 2002). The arrows show the location of the embryonic lethal mutation *st601* and the temperature-sensitive mutation *e286*. (B) The *st601* mutation does not produce the predicted truncated protein fragment. *st601* UNC-45 protein was examined in heterozygous *st601*, N2 and e286 worms by immunoblots with rabbit polyclonal anti-*C. elegans* UNC-45 antibody.

The *st601* mutation results in severely arrested embryonic development (Venolia and Waterston 1990; Fig. 4.3A). *st601* appears to be a null mutation as there is no truncated protein fragment at the expected molecular weight produced in *+/st601* heterozygous worms detected by Western blotting with the polyclonal antibody, which reacts with UCS, TPR(-) and UCS(-) recombinant fragments and full-length UNC-45 (Fig. 4.3B). Furthermore, there is significant published literature on the nonsense-mediated mRNA decay pathway in *C. elegans* and in particular, its muscle cells (Baker and Parker 2004; Conti and Izaurrlade 2005; Hodgkin *et al.* 1989; Pulak and Anderson 1993).

To investigate the consequences of expressing different UNC-45 fragments to rescue *st601* (Fig. 3.2), cloned UNC-45 constructs under control of *unc-54* promoter were injected into the parental heterozygous *st601* hermaphrodite. The dead egg percentage produced by the parental heterozygous *st601* hermaphrodite, was 25% as predicted, based on segregation of embryonic lethal homozygotes (Venolia and Waterston 1990). After injection of the FL construct, the dead egg percentage was decreased. The injection of TPR(-) and UCS constructs also decreased the average *st601* dead egg percentages, but injection of the UCS(-) construct did not change the percentage of *st601* dead eggs (Fig. 4.4). Therefore, the presence of the UCS domain, but not the TPR domain, in FL, TPR(-) and UCS is necessary for significant rescue of *st601* lethality. This result eliminates the dominant-negative hypothesis.

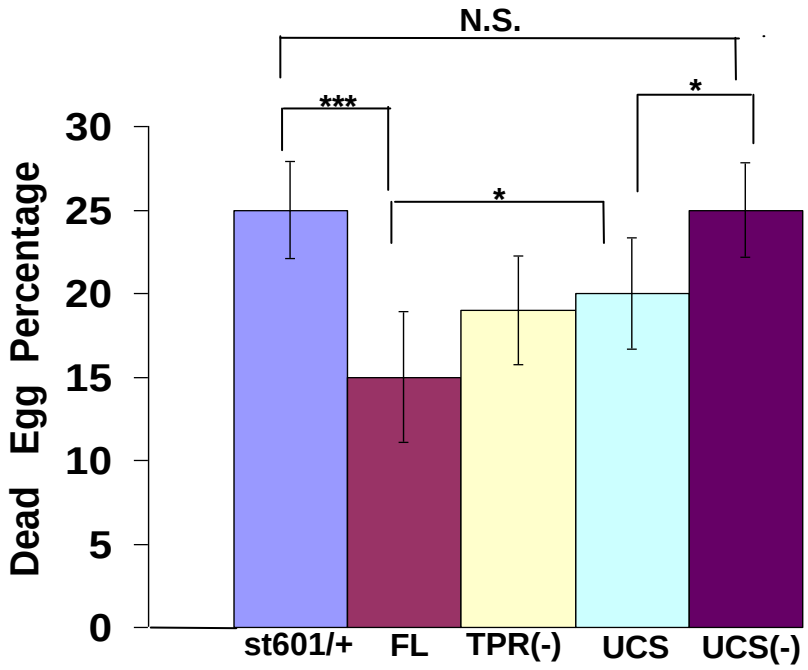


Figure 4.4: Rescue of *st601* mutant by transgenic UCS-containing proteins. The UCS domain and TPR(-) construct are sufficient for significant rescue of *st601* lethality. The percentage of dead eggs of the total progeny was measured after injection of the parental hermaphrodite with the specific constructs. Error bars indicate mean ± s.d. **P*≤0.05; ****P*<0.001. N. S., not significant.

As it is very difficult to evaluate what the actual expression of the full-length and UCS fragments are in the embryos, I cannot rule out the possibility that UCS is less effective than FL with regard the rescue of embryonic lethality of *st601* (Fig. 4.4). An alternative interpretation is that the expression of the UCS fragment is reduced, as in the transgenic experiment with adult nematodes. The loss-of-function temperature-sensitive *e286* mutation permits the further analysis of the potential effects of transgenic expression of UNC-45 fragments on myosin assembly and consequent motility (Barral *et al.* 1998; Epstein and Thomson 1974; Fig. 4.3A). Specific transgenic lines, carrying extrachromosomal arrays to express UNC-45 fragments under control of the strong body-wall muscle specific *unc-54* promoter, were generated by microinjection into the *e286* background. All transgenically expressed proteins have a C-terminal FLAG tag. The results demonstrate that FL, TPR(-), and UCS but not UCS(-) were able to rescue the motility defect of *e286* at the 25 ^{o}C restrictive temperature (Fig. 4.5). *e286* worms grown at 25 ^{o}C moved very slowly. The expression of FL as a positive control rescued the *e286* motility. TPR(-) and UCS were also able to restore the *e286* motility. Transformation with UCS(-) did not change the motility of *e286* worms. Thus, the UCS domain and TPR(-) fragments most significantly rescued *e286* motility at 25 ^{o}C.

Sarcomere assembly in the transgenic *e286* worms grown at 25 ^{o}C was monitored by labeling A bands or their absence with FITC-conjugated mAb 5-6 (anti-MHC A). A bands were detected in transgenic FL, TPR(-) and UCS but not in the UCS(-) or parental *e286* mutant lines (Fig. 4.6).

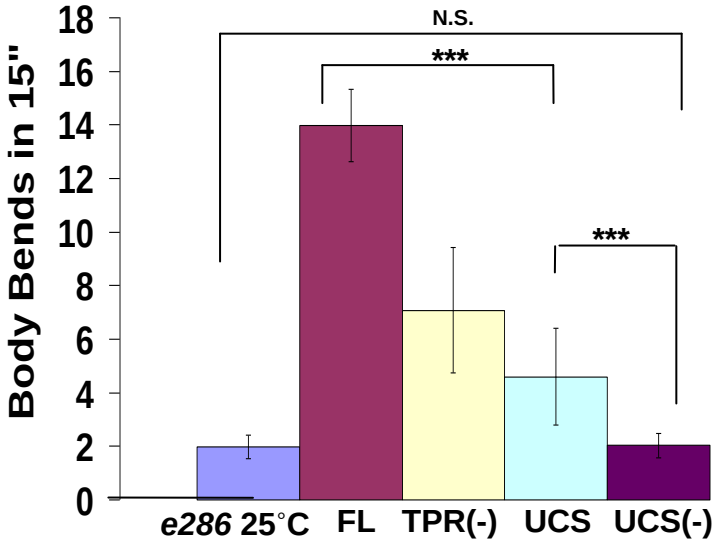


Figure 4.5: Rescue of *e286* motility by transgenic UCS-containing proteins. The UCS domain and TPR(-) construct are sufficient to significantly rescue *e286* motility at 25 °C. The motility of *e286* grown at 25 °C is the negative control and the motility of transgenic FL *e286* is the positive control. Error bars indicate mean ± s.d. ***P<0.001. N. S., not significant.

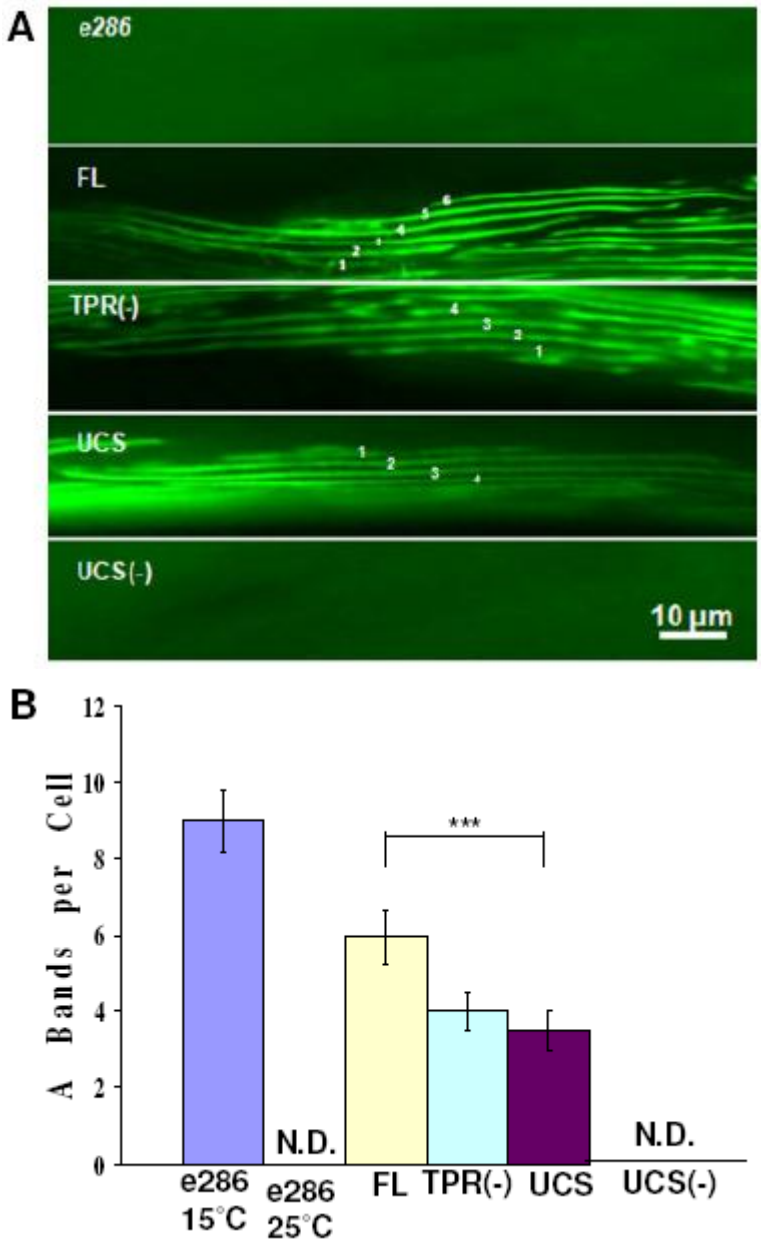


Figure 4.6: Rescue of *e286* A-band assembly by transgenic UCS-containing proteins. (A) The UCS domain and TPR(-) construct rescue A-band assembly in *e286* at 25 °C. The outline of worm muscle cell was faint in the UCS(-) or parental *e286* mutant lines. (D) Quantification of A-band assembly rescue of transgenic *e286* at 25 °C. Error bars indicate mean ± s.d. ***P<0.001.

e286 worms grown at 25 ^{o}C had abnormal thick filament assembly with no detectable myosin organization (Barral *et al.* 1998). The FL protein was able to rescue A-band assembly. The TPR(-) and UCS fragments showed similar rescue of A-band assembly. However, the UCS(-) fragment did not rescue thick filament assembly. In summary, FL, UCS and TPR(-) fragments but not the UCS(-) fragment can rescue A-band assembly in *e286* at the restrictive temperature 25 ^{o}C (Fig. 4.6).

Differences in the extent of rescue of motility and myosin assembly in transgenic *e286* worms could be caused by their differential expression of the fragments or their differential intrinsic activity. The accumulation of these transgenically expressed fragments in *e286* was evaluated by immunoblots with anti-FLAG antibody. Similar to the results presented in the Chapter 3, UCS accumulation was significantly lower than that of the other transgenic products. Densitometric ratios of myosins A and B compared to the loading control pharyngeal myosin D show that accumulation of the body-wall muscle specific myosins A and B in the *e286* background was increased by UCS, TPR(-) and FL but not UCS(-) (Fig. 4.7A).

I also used rabbit polyclonal anti-*C. elegans* UNC-45 antibody for an independent detection of these UNC-45 fragments in transgenic *e286* worms, which further confirmed that UCS protein was expressed in lower amounts (Fig. 4.7B). Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot.

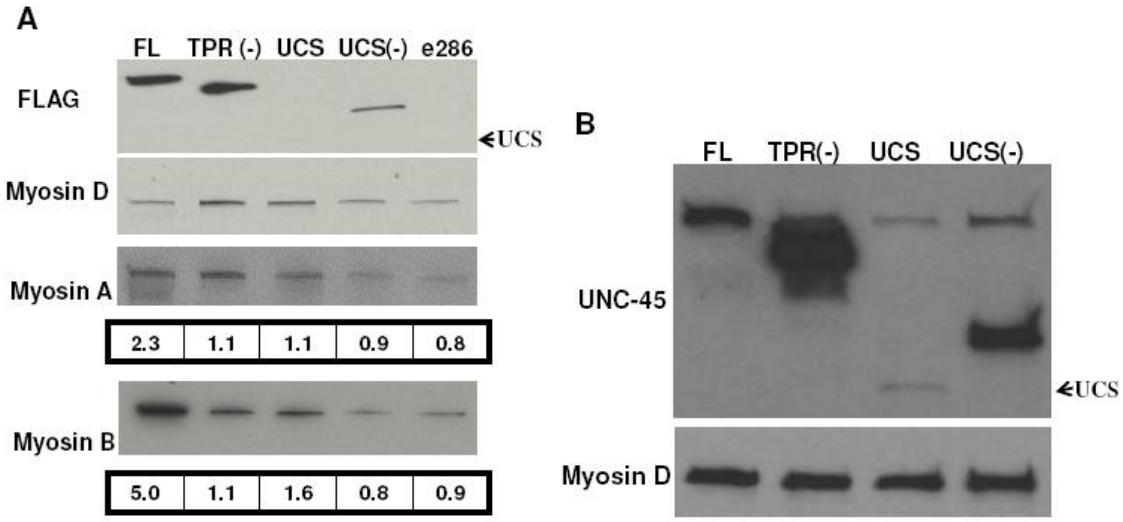
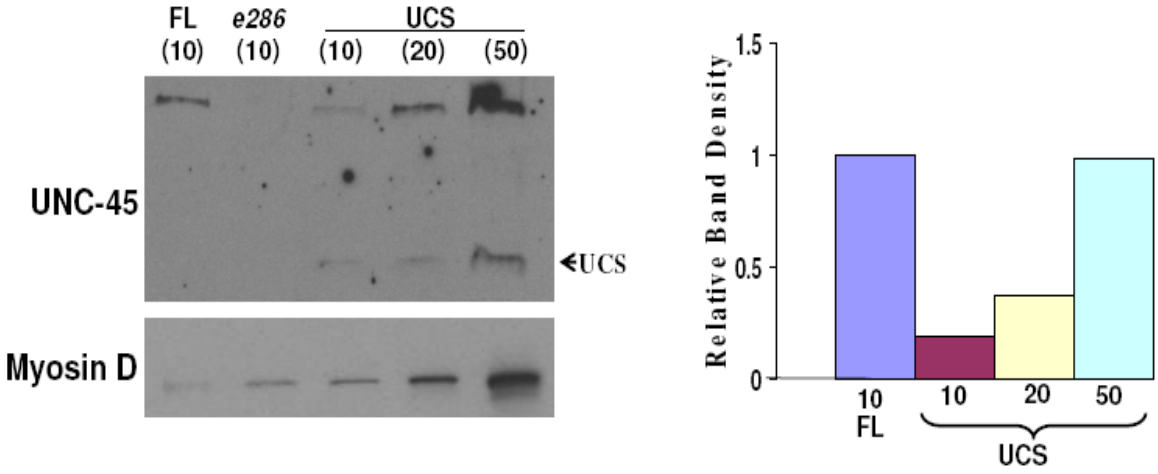


Figure 4.7: The UCS domain is expressed at the lower level compared to the other transgenic proteins and the myosin-binding truncates rescue body-wall muscle myosin accumulation as FL in *e286*. (A) Transgenic expression of UNC-45 fragments was detected by immunoblots with anti-FLAG antibody. UCS band was quite weak with anti-FLAG antibody staining. Body-wall muscle specific myosin heavy chains A and B were detected and quantified by immunoblots. Pharyngeal myosin heavy chain D was used as loading control. (B) The detection of UNC-45 truncates in *e286* by rabbit polyclonal anti-*C. elegans* UNC-45 antibody. UCS and other fragments were detectable on the blots. Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot. Pharyngeal myosin heavy chain D was used as loading control.

The number of transgenic UCS worms in *e286* per lane on immunoblots was modified to semi-quantitatively compare its expression levels with that of FL. Rabbit polyclonal anti-*C. elegans* UNC-45 antibody was employed to provide recognition of all three UNC-45 regions *in vivo* and better detection of the UCS domain (Fig 4.8A). After quantification of the immunoreacted proteins, UCS fragment was found to be expressed at about one fifth of the FL protein level in *e286* worms. The activities ratios of FL versus UCS based on this quantification were calculated (Fig. 4.8B). I quantitatively compared *e286* transformed by either FL or UCS by the expressed protein levels, number of body bends for 15 second intervals, and the A bands assembled per body-wall muscle cell. Although FL is expressed 5 times greater than UCS in the transformed *e286*, its motility in terms of body bend rate is only 3 fold higher, and its A-band assembly is only 1.7 fold higher. These results suggest that FL had about half of the activity compared to UCS on a per molecule basis when expressed in *e286* mutant body-wall muscle. Therefore, UCS is more active in rescuing loss-of-function assembly mutants than the full-length protein. Because FL but not UCS can bind Hsp90, these *e286* results further suggest that actual Hsp90 interaction or that the TPR domain itself may reduce the myosin chaperone activity of the UCS domain in relation to myosin accumulation and its consequent assembly within *C. elegans* muscle.

A



B

Phenotype	Protein Amounts	Body Bends	A Bands
FL/UCS	5/1 (5)	14/4.6 (3)	6/3.5 (1.7)

Figure 4.8: Quantification of the transgenic expression of the UCS domain in *e286* mutant worms. (A) A titration experiment of transgenic UCS worms in *e286* was performed to semi-quantitatively compare the level of UCS expression to that of FL in FL transgenic *e286* by interaction with rabbit polyclonal anti-*C. elegans* UNC-45 antibody. Endogenous UNC-45 and FLAG tagged full-length UNC-45 overlapped on the top of the immunoblot. UCS was visible by polyclonal anti-*C. elegans* UNC-45 antibody. Pharyngeal myosin heavy chain D was used as loading control. (B) The table shows the ratios of protein amounts, body bends, and A bands between transgenic FL and UCS *e286*.

UNC-45 and Hsp90 Compete for Binding to Myosin

Direct binding experiments were performed to distinguish between the effects of Hsp90 and those of the TPR domain upon UNC-45:myosin interaction. A titration of either UNC-45 or Hsp90 binding to a fixed amount of myosin was performed to establish apparent half-maximal binding of UNC-45 or Hsp90 to myosin (Fig. 4.9). The apparent 50% binding of Hsp90 to myosin was at approximately 1.8 μM while that for UNC-45 was at approximately 1.3 μM, based on the quantification accompanying Fig. 4.9.

In order to test the *in vivo* binding of Hsp90 to UNC-45, *suIs2* (integrated lines overexpressing UNC-45) worms were used in the FLAG pull-down experiment. *suIs2* worms overexpresses more UNC-45 in body-wall muscle than transgenic FL wild-type worms carrying extrachromsomal arrays (Landsverk *et al.* 2007). The FLAG pull-down experiment indicates that FLAG tagged UNC-45 can bind Hsp90 in worm lysates, which is consistent with the previously published *in vitro* insect cell lysate pull-down result (Barral *et al.* 2002; Fig. 4.10A). Thus, Hsp90 and UNC-45 have the potential to form complexes *in vivo*.

The titration of recombinant UNC-45 protein with 1.8 μM recombinant Hsp90 protein at a fixed concentration of purified *C. elegans* myosin was then performed in order to investigate the potential effect of the interaction between Hsp90 and the TPR domain on UNC-45 (Fig. 4.10B). SDS-PAGE of the titration of these recombinant *C. elegans* proteins showed that increasing concentration of UNC-45 prevented the binding of Hsp90 to myosin. Quantification of the Coomassie blue staining further showed that the decreases in Hsp90 occur concomitantly with increases in UNC-45, consistent with the two proteins competing with one another for binding to myosin.

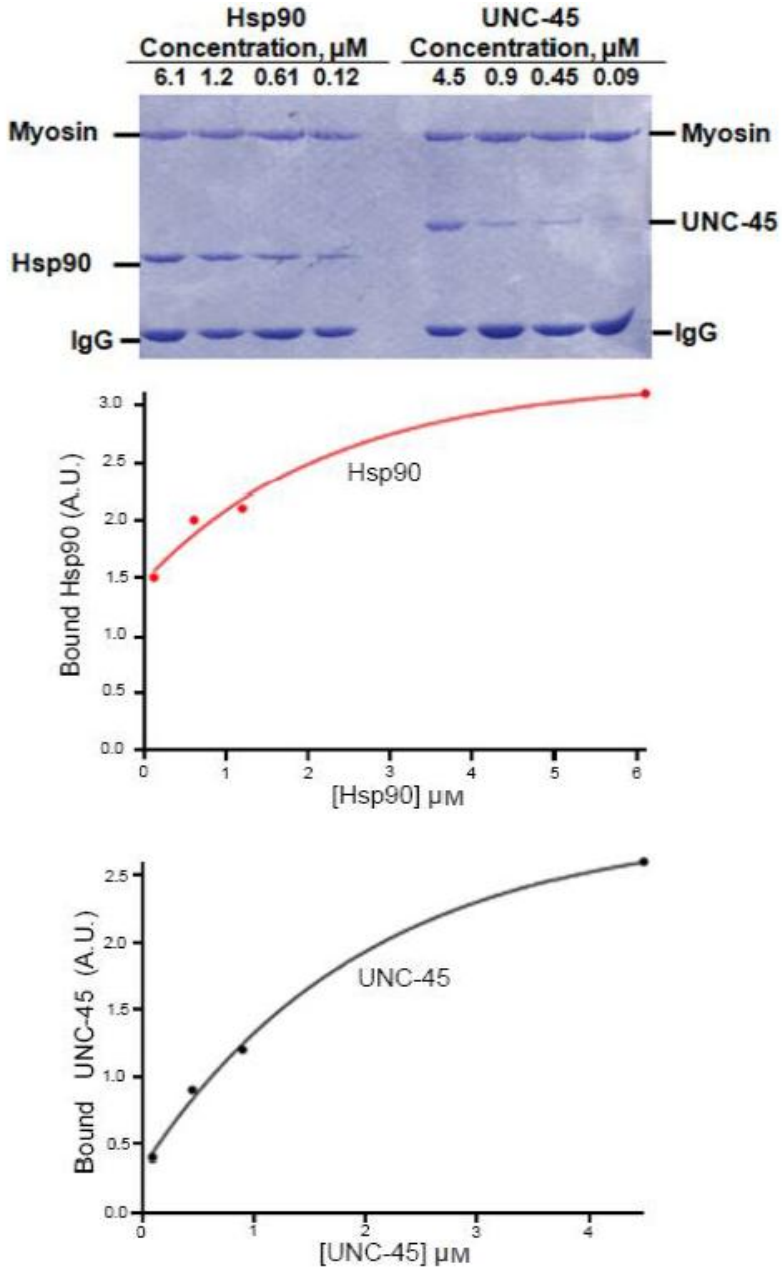
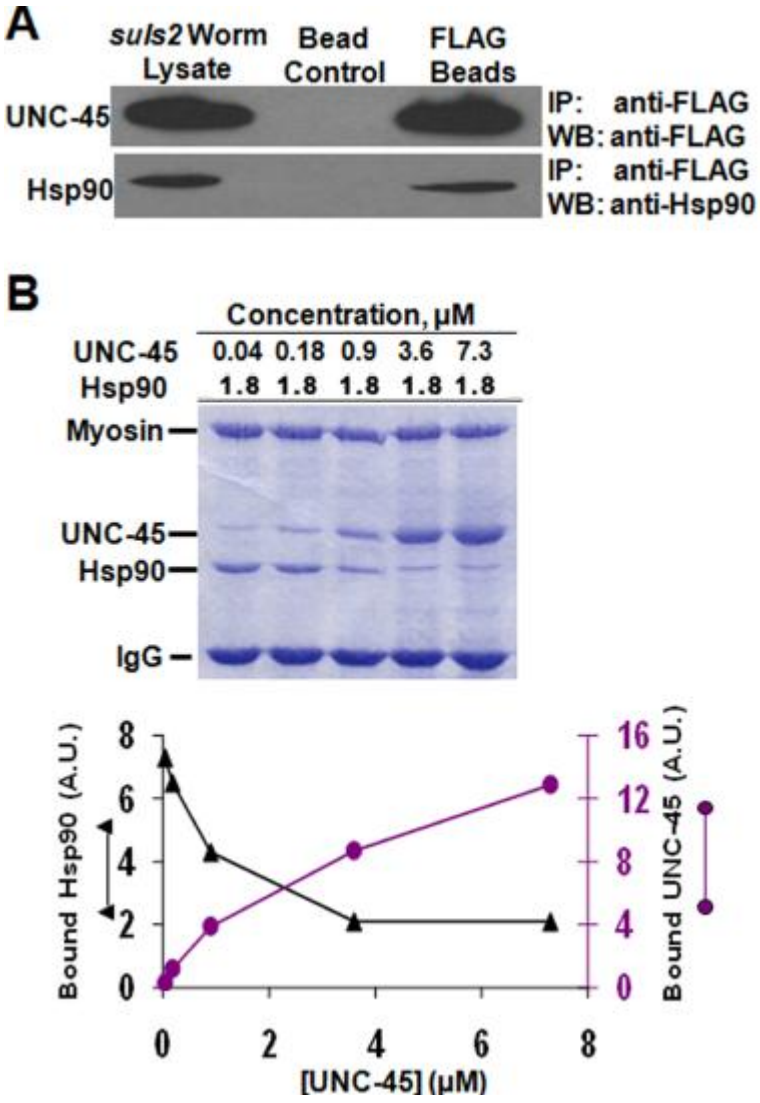


Figure 4.9: Titration of Hsp90 or UNC-45 for myosin binding. A titration of Hsp90 (left side of figure) and UNC-45 (right side of figure) against a fixed concentration of myosin is shown here to test for apparent half-maximal binding of either protein to myosin (done by Alex Hutagalung). The quantification shows that the apparent 50% binding of Hsp90 to myosin was at approximately 1.8 µM while that for UNC-45 was at approximately 1.3 µM. Myosin is bound to beads via antibodies. The purified proteins were detected by Coomassie blue staining.



(Alex H. Hutagalung)

Figure 4.10: Hsp90 and UNC-45 compete for interaction with myosin. (A) UNC-45 interacts with Hsp90. FLAG beads were incubated with *suIs2* worm lysate and myosin-containing complex was eluted with FLAG peptide. Hsp90 and UNC-45 were determined by immunoblotting with anti-FLAG antibody and anti-Hsp90 antibody. (B) A titration of UNC-45 to a fixed amount of Hsp90 at a fixed concentration of myosin is shown here (Alex Hutagalung). The purified proteins were detected by Coomassie blue staining.

DISCUSSION

In Chapter 3, the UCS domain has been shown to possess higher biological activity for myosin accumulation and assembly *in vivo* compared to full-length UNC-45 protein. Full-length UNC-45 can bind the well-known molecular chaperone Hsp90 through its TPR domain whereas the UCS domain can bind myosin but not Hsp90. In this chapter, I propose that in *C. elegans* body-wall muscle, Hsp90 may inhibit rather than activate the myosin chaperoning activity of UNC-45. *In vitro* binding experiments also suggest that UNC-45 and Hsp90 compete for binding to myosin.

In the *st601* experiments, injection of FL and the UCS and TPR(-) fragments rescued the embryonic development of mutant worms. However, UCS(-), the truncated protein missing the UCS domain, did not rescue the homozygous *st601* development at the twofold stage (Fig. 4.4). Therefore, the myosin-binding UCS domain, but not the Hsp90-binding TPR domain, is necessary for normal myosin function at this early developmental stage. This finding rules out the dominant-negative possibility. The explanation for the enhanced activity of the UCS domain compared to FL in wild-type background may be explained through the inhibition by the TPR domain itself or the binding of Hsp90 to the TPR domain.

In the *e286* experiments, the expression of UCS and TPR(-) restored worm motility, A-band assembly and body-wall muscle myosin accumulation of *e286* to an extent approaching the rescue by full-length UNC-45, whereas UCS(-) did not appreciably rescue the temperature-sensitive mutant (Figs. 4.5, 4.6, 4.7). The distinct extents of rescue by UNC-45 protein truncates in *e286* worms could be explained by their differential accumulation (Fig. 4.7). FL levels are 5 fold greater than those of UCS by the titration experiment (Fig. 4.8A). If UCS would be expressed at the same level as FL, its rescue of *e286* would be predicted to be 2 fold greater than that by FL (Fig. 4.8B).

Therefore, the UCS domain not only shows intrinsic biological activity in UNC-45-deficient backgrounds but is more active on a per molecule basis than full-length UNC-45, which can interact with Hsp90.

The pulldown experiment confirms that Hsp90 and UNC-45 interact in *C. elegans* lysates (Fig. 4.10A). The binding experiment with purified recombinant *C. elegans* proteins demonstrates that UNC-45 and Hsp90 compete for myosin. Increasing UNC-45 concentration blocks the binding of Hsp90 to myosin (Fig. 4.10B). Therefore, the interaction between UNC-45 and Hsp90 but not the existence of the TPR domain itself may have an inhibitory effect on myosin binding to UNC-45.

Chapter 5: Conclusions and Future Directions

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The Myosin-Binding UCS domain but not the Hsp90-Binding TPR domain of the UNC-45 Chaperone is Essential for Function in *Caenorhabditis elegans*.

(Weiming Ni, Alex H. Hutagalung, Shumin Li and Henry F. Epstein (2011). *J. Cell. Sci.* 124, 3164-3173)

CONCLUSIONS

UNC-45 was considered as the assembly protein for *C. elegans* sarcomere organization at the very beginning (Epstein and Thomson 1974). Later, people thought that UNC-45 homologs from *C. elegans* to humans may function as putative co-chaperone to target myosins to Hsp90 for folding and subsequent assembly due to the existence of the TPR domain on the N-terminus (Barral *et al.* 1998, 2002; Price *et al.* 2002; Young *et al.* 2003). Recently, it is proposed that UNC-45b may activate Hsp90-dependent myosin folding pathway (Liu *et al.* 2008) and UNC-45 may serve to regulate Hsp90 function on the unfolded myosin motor domain (Mishra *et al.* 2005; Srikakulam *et al.* 2008). Interestingly, UNC-45A has been shown to inhibit the Hsp90-progesterone receptor complex in the maturation pathway (Chadli *et al.* 2006).

Here, I tried to examine whether *C. elegans* UNC-45 is a Hsp90 co-chaperone or *C. elegans* UNC-45 is a myosin chaperone that is regulated by Hsp90. In Chapter 3, it is shown that the UCS domain has the intrinsic biological activity for myosin assembly and accumulation *in vivo*. In Chapter 4, I found that the interaction between the TPR domain and Hsp90 inhibits the function of the UCS domain on myosins *in vivo* and UNC-45 and Hsp90 compete for myosin binding *in vitro*. Therefore, Hsp90 exerts inhibitory regulation on UNC-45 UCS domain's biological activity for myosin assembly and accumulation.

Fig. 5.1 concludes two possible models for UNC-45:Hsp90:myosin interactions in *C. elegans*. UNC-45 may function to target Hsp90 to myosin motor domains as a Hsp90 co-chaperone as in model II of Fig. 5.1 if the N-terminal TPR domain could bind Hsp90 and the C-terminal UCS domain could bind myosin motor simultaneously (Barral *et al.* 2002; Liu *et al.* 2008; Srikakulam *et al.* 2008). However, my results are not consistent with this model. Alternatively, Hsp90 and the UCS domain may not be able to bind

myosin simultaneously because of steric constraints between them when bound to the TPR and UCS domains, respectively. It is possible that Hsp90 and UNC-45 may bind to the same non-native region of the myosin motor domain. The proposed L-shaped structure of the yeast UCS-domain protein She4p which is comparable to the central-UCS fragment that I studied as TPR(-) would be consistent with such constraints (Shi and Blobel 2010). *Drosophila* UNC-45 also possesses a similar structure that may underly these constraints (Lee *et al.* 2011). Therefore, the interaction between Hsp90 and UNC-45 may inhibit binding to myosin as in model I of Fig. 5.1.

The binding of full-length and of UCS to myosin would be predicted to be very similar. Hsp90 inhibition of full-length versus UCS is proposed to be responsible for the differences in per mole activity of FL and UCS *in vivo*. UCS cannot bind Hsp90 and therefore its interaction with myosin is not inhibited by that mechanism. In the presence of Hsp90, the predicted difference would be the result of the formation of binary complexes of Hsp90 and full-length in addition to the competition of both myosin binding whereas UCS would only potentially compete for myosin.

Similar inhibition has been shown in the Hsp90-mediated dissociation of transcriptional regulatory complexes from DNA, diminishing specific transcriptional activity (Freeman and Yamamoto 2002). In addition, Hsp90 inactivates steroid receptors such as glucocorticoid receptor by masking the DNA-binding domain, and the binding of Hsp90 to the hormone-binding domain of the glucocorticoid receptor causes the unfolding of its polypeptide (Eilers *et al.* 1989; Picard *et al.* 1988; Yamamoto *et al.* 1988). The interaction of Hsp90 with glucocorticoid receptor prevents its activation by making DNA binding domain, receptor dimerization sites and nuclear localization sequences of the glucocorticoid receptor inaccessible (Hsu *et al.* 1992; Picard and Yamamoto 1987; Qi *et al.* 1989). Hsp90 also can negatively regulate the activity of this

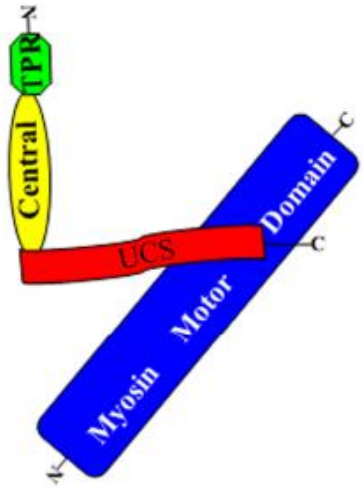
receptor with respect to promoter-dependent transcription (Kang *et al.* 1999). The results of my experiments in *C. elegans* are similar to those with these steroid receptors.

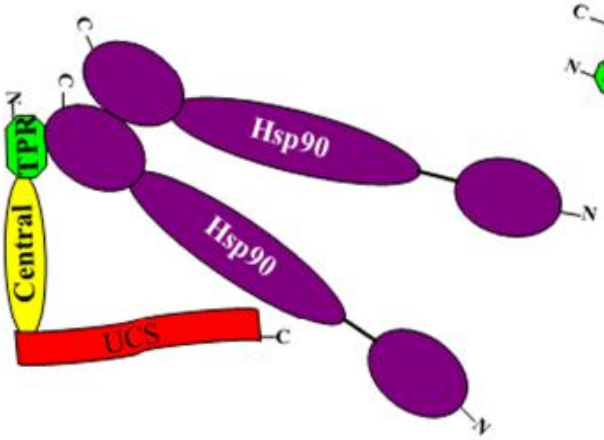
My study shows that the UCS domain possesses intrinsic biological activity *in vivo* and the interaction between TPR domain and Hsp90 inhibits the function of the UCS domain on myosin assembly in *C. elegans* body-wall muscle. My results support the novel finding that Hsp90 may function as an inhibitory regulator of the UNC-45 chaperone, compared to its more usual function as an activating chaperone. Hsp90 may modulate UNC-45 activity to ensure functional levels of myosin accumulation and proper myosin assembly since overexpression can be deleterious as underexpression (Barral *et al.* 1998; Landsverk *et al.* 2007). Hsp90 activity can be regulated by phosphorylation (Mollapour *et al.* 2011; Zhao *et al.* 2001), which may switch on and off its inhibition of UNC-45:myosin interactions for optimal control of myosin accumulation and assembly.

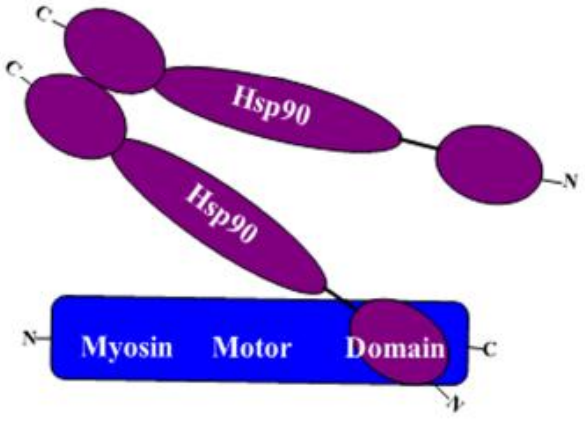
Figure 5.1: Alternative models of UNC-45, Hsp90 and myosin motor interactions. In model I, UNC-45 and Hsp90 compete for myosin motor binding, and the binding of Hsp90 to the TPR domain and of myosin to the UCS domain would be sterically incompatible with one another. In this model, only binary complexes of the combinations of the three proteins would be significant. In model II, UNC-45 and Hsp90 cooperate for myosin motor binding in a ternary complex. Model II would require a major conformational change from the crystallographically based L-shaped structure (Lee *et al.* 2011; Shi and Blobel 2010) to accommodate the binding because the Hsp90 dimer and myosin motor region have molecular weights of 182,000 and 115,000, respectively, which are significantly greater than the 107,000 of UNC-45 (Barral *et al.* 1998, 2002). Hsp90 assumes the open structure because the *in vitro* binding experiments in Figures 4.9 and 4.10B were performed in the absence of ATP. Previous experiments (A. H. Hutagalung and H. F. Epstein, unpublished results) showed that myosin did not bind UNC-45 in the presence of ATP.

Models for UNC-45:Hsp90:Myosin Interactions

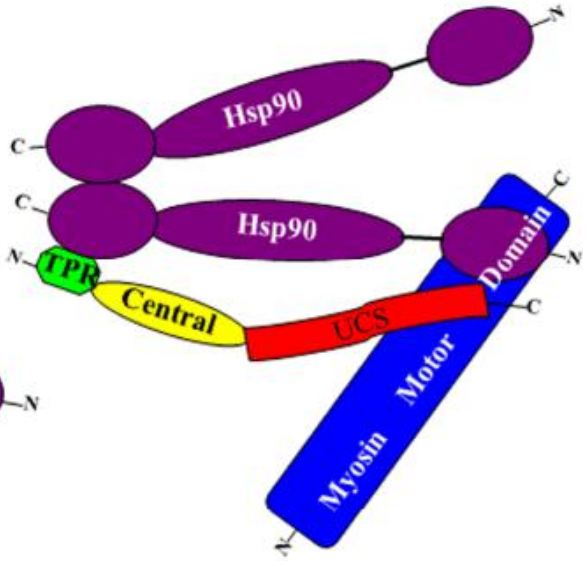
I. Competitive







II. Cooperative



FUTURE DIRECTIONS

The UCS domain has the intrinsic biological activity for myosin assembly and accumulation as discussed in the Chapters 3 and 4. However, it is not known where the active center is localized in the UCS domain for the myosin binding. The alignment of protein sequence for UCS domain-containing proteins shows that there is a highly conserved LTNL sequence in the UCS domain for many organisms (Hutagalung *et al.* 2002; Price *et al.* 2002). The LTNL sequence may be within the myosin-binding site. In order to test this possibility, LTNL in FL UNC-45 or the UCS domain needs to be mutated or deleted. If the deletion or mutation of LTNL in FL UNC-45 will block its ability to rescue *e286* motility, A-band assembly, and myosin accumulation at the restrictive temperature 25^0C, LTNL would be essential for myosin binding. The hypothesis can also be tested in wild-type worms to see whether the deletion or mutation of LTNL in FL UNC-45 will affect its capability of reducing worm motility, A-band assembly, and myosin accumulation.

As previously described in Chapter 1, over 40 dominant-negative mutations were identified in the *C. elegans unc-54* locus (Bejsovec and Anderson 1990). All of the dominant-negative myosin B mutations are localized in the ATP-binding site and actin-binding site (Bejsovec and Anderson 1990). The existence of myosin B mutant protein can cause the complete disruption of thick filament assembly, which is worse than the knockout of myosin B. A potential explanation is that misfolded myosin B mutant proteins sequester chaperones such as UNC-45. Therefore, there are no enough UNC-45 molecules available to correctly fold wild-type myosin A or B proteins. Misfolded myosin B mutant proteins will be degraded and simultaneously unfolded or misfolded wild-type myosin A or B proteins will also be degraded because of the lacking of UNC-45 activity. In order to test this hypothesis, UNC-45 needs to be overexpressed in a

dominant negative myosin B mutant background. If the dominant negative phenotype of myosin B mutant can be rescued by the overexpression of UNC-45, the sequestration of UNC-45 by myosin B mutant proteins will be proved.

As discussed in Chapter 4, the interaction between the TPR domain and Hsp90 inhibits the function of the UCS domain on myosin assembly and accumulation and UNC-45 competes with Hsp90 for myosin binding. According to the competitive model of Fig. 5.1, the binding of Hsp90 to the TPR domain of UNC-45 actually prevents the myosin binding to the UCS domain due to the steric constraints between the TPR domain and the UCS domain. If the interaction between the TPR domain and Hsp90 can be abolished, no competition might occur for binding of UNC-45 and Hsp90 to myosin. There are several ways to disrupt the binding of Hsp90 to UNC-45: deletion of the TPR domain, mutation of the TPR domain, sequestration of the TPR domain by MEEVD peptide, and mutation of the Hsp90 MEEVD motif. If the competition is eliminated with the disruption of the interaction between the TPR domain and Hsp90, it is confirmed that the interaction between the TPR domain and Hsp90 plays an inhibitory role for the UCS domain's function on myosin.

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Vita

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